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Role of cyclooxygenase isoforms and nitric oxide synthase in the modulation of tracheal motor responsiveness in normal and antigen-sensitized guinea-pigs*

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ABSTRACT

The effects of selective cyclooxygenase isoform (COX-1, COX-2) inhibition, alone or in combination with nitric oxide synthase (NOS) blockade, on *in vitro* tracheal muscle responsiveness to histamine were investigated in healthy and ovalbumin (OVA)-sensitized guinea-pigs. Immunohistochemistry showed that COX-1 and COX-2 are constitutively present in normal guinea-pig trachea, particularly in the epithelial layer, and that COX-2 expression is enhanced in OVA-sensitized animals both in epithelial and subepithelial tissues. In normal guinea-pigs, SC-560 (COX-1 inhibitor) or DFU (COX-2 inhibitor) significantly increased the contractile response to histamine, these effects being not additive. NOS inhibition by L-NAME did not affect histamine-induced contraction, but reversed the increase caused by COX-1 blockade, while not modifying the enhancement associated with COX-2 inhibition. In guinea-pigs subjected to OVA-sensitization and challenge, COX-2, but not COX-1, inhibition enhanced the motor responses to histamine, without any influence by L-NAME. In normal, but not in sensitized animals, the removal of epithelial layer from tracheal preparations abolished the enhancing action of DFU on histamine-mediated contraction. A COX-2-dependent release of PGI_2, but not PGE_2, was observed in tracheal tissues from normal and OVA-sensitized guinea-pigs. In conclusion: a) both COX-1 and COX-2 are constitutive in guinea-pig trachea, and COX-2 expression is enhanced by OVA-sensitization; b) in normal animals epithelial COX-2 exerts a PGI_2-dependent inhibitory control on tracheal contractility, and this isoform is subjected to upstream regulation by epithelial COX-1 and NOS, through a complex interplay; c) following antigen sensitization, the inhibitory control on tracheal contractility is maintained by COX-2 induced at subepithelial cell sites.

INTRODUCTION

Both nitric oxide (NO) and prostaglandins (PGs) are relevant mediators in airway physiology and pathology, and their production is regulated by different enzyme isoforms. NO is formed from L-arginine by NO synthase (NOS), of which three isoforms have been identified: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Ogden et al., 1995). PG synthesis depends on cyclooxygenase (COX), two main isozymes of which are currently recognised: COX-1, constitutively expressed in different tissues, and COX-2, regarded as an inducible isoform, since an increase in its expression has been associated with inflammatory processes (Simmons and Botting, 2004).

It is generally believed that the production of NO and PGs by constitutive enzyme isoforms is important for physiological functions, while their formation via inducible isoforms is involved mainly in inflammatory and other pathological responses (Di Rosa et al., 1996). Increasing evidence suggests a link between NOS and COX pathways (Di Rosa et al., 1996), but there is no consensus on the functional consequences resulting from their mutual interactions: NO has been reported to regulate the activity of COX enzymes both by enhancing (Salvemini et al., 1993) and inhibiting (Stadler et al., 1993) their expression or modulating their activity (Di Rosa et al., 1996); on the other hand, a possible influence of PGs on NOS activity has been proposed and current evidence suggests either inhibition (Marotta et al., 1992; Milano et al., 1995), stimulation (Milano et al., 1995) or no interaction (Curtis et al., 1996).

Both NO and COX-derived products influence airway smooth muscle responsiveness to contractile mediators, among which histamine plays a key role in the pathophysiology of allergic responses (Braunstein et al., 1988; Nijkamp et al., 1993). Nevertheless, the possible therapeutic usefulness of NOS and COX inhibitors in asthmatic hyperreactive airways has not been clearly established. Advances in the development of selective COX-1 and COX-2 inhibitors have provided useful tools to analyze the contribution of COX pathways to various pathological conditions. However, selective COX-1 or COX-2 inhibitors have shown unclear effects in airways: a therapeutic effect has been reported for COX-2 inhibitors in allergic inflammation (Oguma et al., 2002) or hyperresponsiveness

of guinea-pig airways after ozone exposure (Nakano et al., 2000), while an increase in airway inflammation and muscle hyperresponsiveness has been observed in sensitized mice when treated with COX-1 or COX-2 inhibitors (Peebles et al., 2002).

The aim of the present study was to determine the influence of COX-1 and COX-2 selective inhibitors, either alone or in combination with a non-selective NOS inhibitor, on *in vitro* tracheal motor responsiveness to histamine, to examine possible interactions between endogenous NO and COX products relevant to the control of airway smooth muscle reactivity. The study was extended from normal to ovalbumin (OVA)-sensitized and OVA-challenged animals, to identify possible changes in the response to pharmacological treatments resulting from the allergic condition.

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METHODS

Male Dunkin–Hartley guinea-pigs (300-400 g b.w.) were employed. The experiments were carried out in accordance with the legislation of Italian authorities (D.L. 27/01/1992 n° 116) which complies with European Community guidelines (CEE Directive 86/609) for the care and use of experimental animals. The experimental protocol was approved by the Animal Care Committee of Pisa University.

Immunohistochemical analysis of COX-1 and COX-2 Tracheo-bronchial tree was removed and placed in phosphate-buffered saline (PBS) maintained at 37°C and oxygenated with carbogen (95% O_2 plus 5% CO_2). Tracheal specimens were immediately fixed in cold 4% paraformaldehyde diluted in PBS overnight. Samples were dehydrated with ethanol, treated with xylene and embedded in paraffin at 56°C. Five-micron-thick serial sections were processed for either routine staining by hematoxylin-eosin or immunostaining.

To perform immunohistochemistry, sections were microwaved in citrate buffer and treated with 1% hydrogen peroxide (H_2O_2) in methanol for 30 min, and normal swine serum (1:20) for 20 min at 37°C. The slides were incubated overnight at 4°C with the following polyclonal primary antibodies: rabbit anti-COX-1 (1:80; code no. 160109; Cayman Chemical Company, Michigan, USA); rabbit anti-COX-2 (1:2000; code n° ALX 210711, Alexis Biochemicals, Lausen, Switzerland). Primary immunoglobulins were diluted in PBS with 0.1% bovine serum albumin and 0.1% sodium azide. The immunoprecipitates were detected by an indirect streptavidine-peroxidase method (Dakopatts, Glostrup, Denmark) as previously reported (Bernardini et al., 1999; Virdis et al., 2005). Briefly, sections were sequentially exposed to biotinylated secondary immunoglobulins and peroxidase-labeled streptavidin complex. The slides were then treated with 3,3'-diaminobenzidine tetra-hydrochloride (DAB, 1 mg/ml Tris buffer (Dakopatts Glostrup, Denmark) containing 0.02% H_2O_2 and counterstained with haematoxylin (1:30). All reactions were carried out at room temperature in a humidified chamber and PBS was used for washes between each step. Negative controls were obtained by omitting primary antibodies or substituting the primary antibody with rabbit preimmune serum. Specificity controls included preadsorption of anti-COX-1 and -COX-2 primary

antibodies with the corresponding blocking peptides, COX-1 (Cayman Chemical Company, cod. no. CAY 360109) and COX-2 (Alexis Biochemicals, cod. no. ALX 153-063), respectively. To test endogenous peroxidases and avidin-binding activity, slides were incubated with DAB alone or streptavidin-peroxidase complex plus DAB, respectively.

Specimens of six animals from different experimental group were immunostained for COX-1 and COX-2 and then blindly evaluated, using light microscopes, by two investigators (N.B., C.I.) who were unaware of other results. The intensity of immunostaining was ranked as: – (absent), + (weak), ++ (medium), +++ (strong) as previously reported (Fux et al., 2005).

Antigen sensitization and challenge OVA-sensitization was induced as previously described (Nieri et al., 1992). Briefly, two consecutive administrations of OVA saline solution (100 mg/kg i.p. + 100 mg/kg s.c.) were performed 14-19 days before *in vitro* experiments. A group of sensitized guinea-pigs were exposed to OVA aerosol (1% in saline) in a box connected to an ultrasonic nebulizer (Artsana, Mod. Project 38080), on days 7, 14, 15 and 16 from sensitization. The aerosol was generated for 10 min, or less if respiratory distress occurred. Respiratory distress was defined as the onset of sneezing, cough and/or indrawing of thoracic wall. Sham-challenged guinea-pigs were exposed to aerosols with saline solution without OVA. Animals were sacrificed for tracheal removal 6 h after the last challenge. Multiple antigen-challenged guinea-pig is a model which reproduces several of the characteristic features of asthma, including bronchial hyperreactivity and inflammation (Samb et al., 2001). The presence of airway hyperreactivity to histamine was confirmed 6 h after last antigen challenge by preliminary experiments in anaesthetized guinea-pigs, recording pulmonary inflation pressure as an index of airway resistance (data not shown).

***In vitro* recording of tracheal smooth muscle contraction** Guinea-pigs were sacrificed by cervical dislocation and bleeding under light ether anaesthesia. The tracheo-bronchial tree was immediately removed and placed in Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7; $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.5). A set of experiments was carried out in epithelium-deprived tracheae, after removing the epithelial layer by rubbing the internal surface of tracheal tube with the edge of a small spatula. Each specimen, examined by

histology, revealed at least 70% removal of epithelium. Four multiple-ring preparations were obtained from a single tracheal tube. Each tracheal preparation was suspended, under a resting tension of 0.5 g, in a 20-ml organ bath containing the above saline solution maintained at 37°C and oxygenated with carbogen (95% O_2 plus 5% CO_2). Isometric contractile responses were recorded via a force-displacement transducer (mod. FT03D, Grass Instruments, UK) connected to a polygraph (mod. WR 3101, Graphtec Corp., Japan). After an equilibration period of 10 min, a reference contraction to KCl 40 mM was obtained. After tone recovery by repeated washings with fresh Krebs-Henseleit solution, SC-560 (COX-1 inhibitor), DFU (COX-2 inhibitor), SC-560 + DFU or L-NAME (non-selective NOS inhibitor), or a combination of one COX inhibitor with the NOS inhibitor were added to the organ bath and incubated for 30 min. Thereafter, a single cumulative concentration-response curve to histamine (10^{-7}-10^{-4} M) was constructed. Both SC-560 and DFU were tested at 10^{-6} M in agreement with selectivity studies (Smith et al., 1998; Riendeau et al., 1997). The effects of test drugs were compared with those obtained with their vehicle (controls). In each tracheal preparation retrieved from sensitized guinea-pigs, OVA 10^{-5} M was added at the end of experiment and the respective muscle contraction was recorded.

Assay of prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) release PGE_2 and PGI_2 release from tracheal preparations in response to histamine was assessed, both in normal and OVA-sensitized guinea-pigs, to investigate their role in the modulation of tracheal contractile activity by COX-1 and COX-2 isoenzymes. Isolated tracheal rings were incubated using a 24-well plate at 37°C. Each well contained three tracheal rings in 0.5 ml of carbogenated Krebs-Henseleit buffer. Fifteen rings were isolated from each trachea, and therefore five different conditions per preparation could be tested. PGE_2 or PGI_2 levels were measured after histamine incubation, in the absence or in the presence of SC-560 (10^{-6} M) or DFU (10^{-6} M), either alone or in combination. The experimental design was the same as that employed for contraction recording, but histamine was applied only at the highest concentration (10^{-4} M). The supernatant was collected after 20-min incubation with histamine and stored at -70°C until assay. PGE_2 was assayed as such, while PGI_2 was measured as its stable hydrolysis product 6-keto-$PGF_{1\alpha}$. In both cases, an enzyme immunoassay was utilized, in

accordance to the manufacturer's instructions (Cayman Europe, Tallinn, Estonia). The results are reported as pg of PG per mg of tissue weight.

Drugs and chemicals Histamine dihydrochloride, L-N^G-nitro-arginine methylester, ovalbumin (grade III), and SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole] were obtained from Sigma-Aldrich (St. Louis, MO, USA); DFU [5,5-dimethyl-3(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone] was kindly provided by Merk Frosst Canada Inc. (Dorval, Canada). L-NAME, histamine and KCl were dissolved in Krebs-Henseleit solution; DFU and SC-560 were dissolved in dimethylsulphoxide (DMSO 10^{-2} M). Dilutions of stock solutions were freshly prepared in Krebs Henseleit solution for all drugs at the day of experiment. Concentration of DMSO in the bath was always below 0.01 % v/v.

Data analysis In vitro responses to histamine, obtained in the presence or in the absence of test drugs, were expressed as percentage of active tension induced by 40 mM KCl in the same preparation. All data were reported as mean ± S.E. of n experiments. Comparisons between means was performed by Student t test for unpaired data (two groups) or analysis of variance (ANOVA) (more than two groups) followed by Bonferroni post test. P values <0.05 were considered significant.

RESULTS

Immunohistochemical evaluation of COX-1 and COX-2 expression Both COX-1 and COX-2 are constitutively expressed in normal guinea-pig trachea. In particular, COX-1 was found in the columnar cells of pseudostratified ciliated epithelium, which showed intense apical immunostaining at the luminal surface, corresponding to the ciliar layer, but scanty cytoplasmic staining (Fig. 1A). No specific immunoreaction was observed in subepithelial connective and muscle tissues (Figs. 1A, 2A). COX-2 was widely expressed in cytoplasm of columnar epithelial cells and in subepithelial resident inflammatory cells localized in lamina propria (Fig. 1C); low amounts of specific anti-COX-2 immunoreaction were detected in tracheal condrocytes and smooth muscle cells of normal animals (Figs. 1C, 2C).

Tracheal tissues from OVA-sensitized animals showed different patterns of COX isoform expression compared to normal ones. The epithelium, which appeared thickened, with mucous metaplastic changes and extensive alteration at luminal surface, showed weak cytoplasmic COX-1 expression with scattered epithelial cells more intensely immunostained (Fig. 1B). Subepithelial tissues, including lamina propria and submucosa as well as tracheal cartilage (Figs. 1B, 2B) and smooth muscle (Fig. 2B), did not express detectable COX-1 staining. By contrast, COX-2 expression was markedly increased in tracheal specimens excised from OVA-sensitized animals. Large amounts of COX-2 were indeed detected in the epithelial layer, mainly in the cytoplasm of basal cells and columnar cells in their apical portion (Fig. 1D). COX-2 was also found to be intensely expressed in subepithelial inflammatory cells and vessels of lamina propria (Fig. 1D) as well as in tracheal smooth muscle cells and condrocytes (Fig. 2D). Preadsorption of primary antibodies with the respective blocking peptides completely abolished the staining either in normal or sensitized trachea (data not shown). The degree of COX-1 and COX-2 immunoreactivity in tracheal samples from normal and sensitized animals is summarized in table 1.

In vitro recording of smooth muscle contraction The responses to KCl 40 mM, used as an internal standard contracturant, were not significantly different among the experimental groups (298.6±32.13 mg tension, n=75 for normal guinea-pigs; 297.2±24.60 mg tension, n=70 for OVA-

sensitized guinea-pigs; 275.1±27.27 mg tension n=34 for OVA-challenged guinea-pigs). SC-560, DFU or L-NAME did not significantly modify the basal tone of tracheal preparations both in normal and sensitized guinea-pigs, although in a few cases a slight loss of basal tone was observed with COX inhibitors.

Normal guinea-pigs Histamine (10^{-7}-10^{-4} M) induced concentration-dependent contractions which were significantly enhanced by COX-1 and COX-2 inhibitors (Fig. 3A,B). The increment of maximal contraction was 40.0% for SC-560 (10^{-6} M) and 32.3% for DFU (10^{-6} M). A similar enhancing effect was obtained upon concomitant application of SC-560 and DFU (increment of maximal contraction: 33.0%) (Fig. 3C). Both COX inhibitors, while enhancing the contractile response to histamine, did not modify its potency, with pD2 values ranging from a minimum of 5.18±0.23 in DFU control group (n=7) to a maximum of 5.54±0.37 in SC-560-treated group (n=8). The NOS inhibitor L-NAME (10^{-4} M) did not influence the tracheal responsiveness to histamine, but suppressed the enhancing effect of SC-560 (Fig. 4A). In addition, the enhancing action of DFU remained largely unaffected by L-NAME (Fig. 4B), and the moderate decrease in the effect of DFU, occurring at histamine concentrations below 10^{-6} M (Fig. 3B and 4B), was likely to reflect the biological variability of experimental settings.

OVA-sensitized guinea-pigs Tracheal preparations from guinea-pigs sensitized to OVA were used to evaluate whether antigen sensitization affects the influence of COX isoforms on airway muscle response to histamine. Under these conditions, the responsiveness to histamine was not significantly affected by SC-560 (10^{-6} M), while a significant enhancement was observed with DFU (10^{-6} M) (increment of maximal response: 23.6%) (Fig. 5A,B). L-NAME (10^{-4} M) did not significantly influence the control response to histamine, the enhancement induced by DFU, and the contractions evoked by histamine in the presence of SC-560 (Fig. 5C,D).

OVA-challenged guinea-pigs Although in vivo airway hyperresponsiveness to histamine was observed 6 h after the last aerosol with OVA (data not shown), in vitro experiments did not show any tracheal hyperreactivity to the autacoid, according to findings obtained by Mitchell et al. (2004) in pig bronchi. Fig. 6A shows that the contractile activity evoked by histamine was enhanced by

DFU (10^{-6} M) (increment of maximal contraction: 37.1%), while it was not modified by SC-560 (10^{-6} M). L-NAME did not affect the histamine-induced contraction when assayed alone or in the presence of either COX-1 or COX-2 inhibitor (Fig. 6B). As already observed for normal animals, in the presence of L-NAME the enhancing action of DFU on the contractile responses to histamine, in tracheal preparations from OVA-challenged guinea-pigs, was not apparent at histamine concentrations lower than 10^{-6} M (fig. 6B).

Epithelium-denuded trachea from normal and OVA-sensitized guinea-pigs In epithelium-denuded tracheal preparations from normal guinea-pigs, DFU (10^{-6} M) did not significantly affect the concentration-response curve to histamine (Fig. 7A). By contrast, in OVA-sensitized animals, histamine-induced contraction was significantly enhanced by DFU (increment of maximal contraction: 26.4%) (Fig. 7B). Epithelium removal prevented also the enhancing action of SC-560 (10^{-6} M) on histamine-induced contraction in normal animals. In addition, as already observed in preparations with intact epithelium, COX-1 blockade did not modify the responsiveness to histamine following epithelial deprivation of tracheal tissues from OVA-sensitized guinea-pigs (data not shown).

PGE_2 and PGI_2 release A similar basal release of PGE_2 was measured from normal and OVA-sensitized guinea-pig trachea (table 2). Following tracheal stimulation with histamine (10^{-4} M) for 20 min, an increase in PGE_2 production was observed in both cases, with a greater increment in preparations from normal animals. SC-560 (10^{-6} M) significantly inhibited histamine-induced PGE_2 release when applied to tracheal specimens isolated from normal and OVA-sensitized animals, whereas DFU (10^{-6} M) was without effects (table 2). At variance with PGE_2, basal PGI_2 release was significantly higher in tracheal tissues from OVA-sensitized guinea-pigs (table 2). A significant increment of PGI_2 content was detected in the medium after incubation of tracheal specimens with histamine, both in normal and OVA-sensitized animals. Both COX-1 and COX-2 blockade was associated with a decrease in histamine-induced PGI_2 release, the most prominent inhibition being observed upon exposure of tracheal preparations from OVA-sensitized guinea-pigs to DFU (table

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2). Concomitant incubation with SC-560 and DFU lowered PGI_2 levels towards values detected in normal animals under basal conditions (table 2).

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DISCUSSION

The role of COX isozymes in the modulation of airway smooth muscle in healthy subjects is currently debated. Although various authors reported the absence of COX-2 expression in the tracheo-bronchial system of normal guinea-pigs (Amann et al., 2001; Oguma et al., 2002), the role of COX-2 has been proposed to be relevant in the regulation of tracheal intrinsic tone in this species (Charette et al., 1995), and a constitutive COX-2 expression has been reported in normal airways (Schlemper et al., 2005). Our results from normal guinea-pigs, showing that DFU enhances histamine-induced contraction, indicate a functional role for COX-2 isoform which appears to mediate an inhibitory control on airway muscle. It can be excluded that COX-2 activity in normal trachea depends on inducing stimuli linked to surgical manipulation of isolated tissues, since COX-2 expression was clearly revealed by immunohistochemistry in tracheal tissues immediately after excision. Therefore, our data suggest that COX-2 expression in airways does not occur exclusively in inflamed or manipulated tissues and that this isozyme regulates the contractile activity of tracheal smooth muscle under normal conditions.

In the present study, a functional activity, similar to that observed for COX-2, was shown for the COX-1 isoform in normal tissues, where the selective COX-1 inhibitor SC-560 enhanced histamine-induced tracheal contractions. An inhibition of the release of relaxant PGs from the epithelial compartment (Prié et al., 1990) is the most likely mechanism explaining tracheal muscle hyperresponsiveness to histamine after blockade of COX activities in normal animals. In support of this view, our experiments showed that immunostaining of both COX isoforms was prevalent in the epithelial layer, and that the DFU-induced enhancement of histamine-induced contraction was abolished in epithelium-deprived tracheal preparations.

At variance with COX inhibitors, L-NAME *per se* did not modify histamine-induced contraction. In this respect, controversial findings have been reported about the effect of NOS inhibition on airway responsiveness to spasmogens. In guinea-pig trachea with intact epithelial layer, a weak potentiating effect by L-NAME in the proximal, but not distal, portion has been shown (Kloek et al., 2002). Moreover, the inability of L-NAME to modulate murine tracheal contractile responses to

carbachol has been described by Kakuyama et al. (1999), who observed a L-NAME-induced small increase (about 15%) of maximal response to the cholinergic agonist only when NOS inhibition was tested in the presence of indomethacin. Other authors reported a NO-mediated relaxing effect counterbalancing *in vitro* tracheal contraction to spasmogens (Nijkamp et al., 1993), and the use of indomethacin in the organ bath throughout the whole experiment was hypothesised as the cause underlying the L-NAME-induced effect (Kakuyama et al., 1999). Other factors might be responsible for the conflicting results from different laboratories. For instance, open tracheal preparations (i.e. rings and strips) have been suggested to be insensitive to NO-mediated relaxation, since epithelial-derived NO diffuses away in the medium, thereby decreasing its concentration in proximity of smooth muscle (Folkerts and Nijkamp, 1998). Moreover, circadian fluctuations of NOS activity have been described in many tissues, including lungs (Tunctan et al., 2002), and a different metabolism of NO can give rise to formation of reactive molecular species modulating smooth muscle reactivity (Sadeghi-Hashjin et al., 1996), and even COX activity (Landino et al., 1996).

Of note, in the present study there was a lack of additivity of the motor enhancing effects exerted by SC-560 and DFU. Moreover, L-NAME, while being ineffective when assayed alone, prevented the stimulant action of SC-560 on histamine-induced contraction, without modifying the potentiating effect of DFU. These findings indicate that both COX isoforms and NOS operate within the same regulatory pathway at level of the tracheal epithelial compartment, and that a complex interplay between COX-1, NOS and COX-2 is likely to exist. In particular, as illustrated schematically in Fig. 8A, our data suggest that: a) epithelial COX-2 exerts an inhibitory influence on tracheal smooth muscle responsiveness; b) NOS may exert an inhibitory control on COX-2, but such a modulating action is prevented by COX-1 which operates a tonic inhibition on NOS activity. In line with this functional model, the enhancing contractile effect observed with SC-560 may result from the removal of an inhibitory control of COX-1 on NOS activity, with subsequent NOS activation and onset of a NO-mediated inhibition on constitutive COX-2 function, leading to a reduced formation of relaxant COX-2-derived prostanoid/s. Consistently with this view, inhibitory actions of COX-1

products on NOS activity have been reported in airways (Kakuyana et al., 1999) and other tissues (Marotta et al., 1992; Illiano et al., 1996), and a COX-2 inhibition by NOS pathway has been previously demonstrated by various authors (Mollace et al., 2005).

In OVA-sensitized guinea-pigs, the histamine contractile activity was differently modulated by COX inhibitors, since only DFU, but not SC-560, enhanced the autacoid-induced contraction. These data suggest the occurrence of changes in the respective roles of COX isozymes in the control of airway smooth muscle reactivity, as a consequence of allergic sensitization. The reduced significance of COX-1 isoform in the modulation of histamine-induced contraction, following OVA-sensitization, might depend on the specific localization of this isoform in the epithelial ciliar layer (instead of cytoplasmic compartment as observed for COX-2), which undergoes extensive alteration in sensitized animals, as shown by immunohistochemistry.

With regard to the role played by NOS pathway in sensitized animals, it does not appear to be significant in this setting, since L-NAME did not modify the responsiveness of tracheal preparations to histamine both under control conditions and in the presence of COX-1 or COX-2 blockade. A direct relaxant effect by NO on airway smooth muscle has been excluded also by Larsson et al. (2005) in lung parenchimal strips from sensitized guinea-pigs, where the Authors revealed an indirect protective effect of NO on antigen-induced contraction via inhibition of leukotrienes release from mast cells.

The loss of influence of COX-1 and NOS pathways on COX-2-mediated control of airway muscle responsiveness, taken together with the observation that the enhancing effect of DFU on histamine-induced contractions persisted after epithelium-deprivation of tracheal preparations, suggests that, in antigen-sensitized guinea-pigs, the contractile responsiveness to histamine is modulated by COX-2 at extra-epithelial sites, and that the regulatory network of COX isoforms and NOS at epithelial level looses its functional significance in this setting (Fig. 8B). In support of this proposal, we obtained immunohistochemical evidence that, following OVA-sensitization, COX-2 expression was markedly increased at subepithelial level, particularly in smooth muscle cells.

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A number of different agents, including histamine, have been reported to induce the release of relaxant PGs, namely PGE_2 and PGI_2, in airways, and the inhibition of their release is regarded as a possible cause of tracheobronchial hyperresponsiveness elicited by COX inhibitors (Folkerts and Nijkamp,1998; Carey et al., 2003). To investigate the role of these prostanoids in the present contractile effects elicited by COX-1 and COX-2 inhibitors, we assayed PGE_2 and PGI_2 release from isolated tracheal preparations. Both in normal and in OVA-sensitized animals, histamine-induced PGE_2 release was not affected by COX-2 blockade, while being inhibited only by SC-560. However, this observation, indicating COX-1 as the source of PGE_2 release in airways, is consistent with previous findings by Gavett et al. (1999) in mice, where a constitutive expression of both COX-1 and COX-2 in control lung was present, but PGE_2 in bronchoalveolar lavage fluid decreased only in COX-1 knockout animals. When considering PGI_2, both COX isoforms appear to contribute to its release from tracheal specimens of normal animals, whereas in OVA-sensitized guinea-pigs COX-2 accounted mostly for histamine-induced PGI_2 release, these observations being consistent with immunohistochemical data. These findings, taken together with the results of our experiments on tracheal contractility, suggest that PGI_2, but not PGE_2, is responsible for COX-2-mediated modulation of tracheal responsiveness both in normal and OVA-allergic guinea-pigs. In support of such proposal, previous studies have shown that PGI_2 exerts relaxant effects on airway smooth muscle and can be released from tracheal tissues (Prié et al., 1990). In addition, the COX-2 pathway has been reported to mediate PGI2 production also in smooth muscle cells of extra-respiratory sites, including the vascular system (Schildknecht et al., 2004).

Tracheal preparations from antigen-challenged guinea-pigs did not display substantially different response patterns with respect to animals subjected only to OVA-sensitization, indicating that, in our experimental model, sensitization alone is able to determine alterations in tracheal responsiveness to COX and NOS inhibitors which persist in the asthma model of antigen-challenged guinea-pig. The absence of a NO-mediated influence on the control of smooth muscle responsiveness in asthmatic airways has been already reported in guinea-pigs as a result of arginase activity up-regulation (Meurs et al., 2002) or nNOS protein down-regulation (Samb et al., 2001).

Therefore, our data extend the available evidence indicating a lack of influence by NO on the control of airway smooth muscle responsiveness in multiple antigen-challenged animals, in the presence of COX isoform inhibition.

In conclusion, the main findings obtained in the present work show that: a) both COX-1 and COX-2 isoforms are constitutively expressed in guinea-pig trachea and their inhibition induces tracheal hyperresponsiveness to histamine in normal guinea-pigs, while in antigen-sensitized and challenged animals only COX-2 inhibition is able to evoke such enhancing action; b) in normal guinea-pigs, the NOS pathway mediates a complex interplay between COX-1 and COX-2 at epithelial level, where COX-1 inhibits NOS, thus preventing the inhibitory action of NOS on COX-2; c) after antigen-sensitization, the epithelial COX-1/NOS/COX-2 network is impaired and COX-2 isoform, upregulated at subepithelial level, particularly in smooth muscle cells, exerts its modulatory function through the release of PGI_2.

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REFERENCES

Amann R, Schuligoi R and Peskar BA (2001) Effects of COX-1 and COX-2 inhibitors on eicosanoid biosynthesis and the release of substance P from the guinea-pig isolated perfused lung. *Inflamm Res* **50**:50-53.

Bernardini N, Bianchi F and Dolfi A (1999) Laminin and beta1 integrin distribution in the early stages of human kidney development. *Nephron* **81**:289-295.

Braunstein G, Labat C, Brunelleschi S, Benveniste J, Marsac J and Brink C (1988) Evidence that the histamine sensitivity and responsiveness of guinea-pig isolated trachea are modulated by epithelial prostaglandin E2 production. *Br J Pharmacol* **95**:300-308.

Carey MA, Germolec DR, Langenbach R, Zeldin DC (2003) Cyclooxygenase enzymes in allergic inflammation and asthma. *Prostaglandins Leukotrienes Essent Fatty Acids* **69**: 157-162.

Charette L, Misquitta C, Guay J, Reindeau D and Jones TR (1995) Involvement of cyclooxygenase 2 (COX-2) in intrinsic tone of isolated guinea pig trachea. *Can J Physiol Pharmacol* **73**:1561-1567.

Curtis JF, Reddy NG, Mason RP, Kalyanaraman B and Eling TE (1996) Nitric oxide: a prostaglandin H synthase 1 and 2 reducing cosubstrate that does not stimulate cyclooxygenase activity or prostaglandin H synthase expression in murine macrophages. *Arch Biochem Biophys* **335**:369-376.

Di Rosa M, Ialenti A, Ianaro A and Sautebin L (1996) Interaction between nitric oxide and cyclooxygenase pathways. *Prostaglandins Leukotrienes Essent Fatty Acids* **54**:229-238.

Folkerts G, Kloek J, Muijsers RB and Nijkamp FP (2001) Reactive nitrogen and oxygen species in airway inflammation. *Eur J Pharmacol* **429**:251-262.

Folkerts G and Nijkamp FP (1998) Airway epithelium: more than just a barrier. *Trends Pharmacol Sci* **19**:334-341.

Fux R, Schwab M, Thon K-P, Gleiter C H and Fritz P (2005) Cyclooxygenase-2 expression in human colorectal cancer is unrelated to overall patient survival. *Clin Cancer Res* **11**:4754-4760.

Gavett SH, Madison SL, Chulada PC, Scarborough PE, Qu W, Boyle JE, Tiano HF, Lee CA, Langenbach R, Roggli VL and Zeldin DC (1999) Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest* **104**:721-732.

Illiano S, Marsault R, Descombes JJ, Verbeuren T and Vanhoutte PM (1996) Regulation of nitric oxide-like activity by prostanoids in smooth muscle of the canine saphenous vein. *Br J Pharmacol* **117**:360-364.

Kakuyama M, Ahluwalia A, Rodrigo J and Vallance P (1999) Cholinergic contraction is altered in nNOS knockouts. Cooperative modulation of neural bronchoconstriction by nNOS and COX. *Am J Respir Crit Care Med* **160**:2072-2078.

Kloek J, Nijkamp F, Bloksma N, De Clerck F and Folkerts G (2002) Differential responsiveness of proximal and distal parts of isolated guinea pig trachea. *Eur J Pharmacol* **442**:289-294.

Landino LM, Crews BC, Timmons MD, Morrow JD and Marnett LJ (1996) Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci* USA **93**:15069-15074.

Larsson AK, Back M, Hjoberg J and Dahlen SE (2005) Inhibition of nitric-oxide synthase enhances antigen-induced contractions and increases release of cysteinyl-leukotrienes in guinea pig lung parenchyma: nitric oxide as a protective factor. *J Pharmacol Exp Ther* **315**:458-465.

Marotta P, Sautebin L and Di Rosa M (1992) Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line J774. *Br J Pharmacol* **107**:640-641.

Meurs H, Mckay S, Maarsingh H, Hamer MA, Macic L, Molendijk N and Zaagsma J (2002) Increased arginase activity underlies allergen-induced deficiency of cNOS-derived nitric oxide and airway hyperresponsiveness. *Br J Pharmacol* **136**:391-398.

Milano S, Arcoleo F, Dieli M, D'agostino R, D'agostino P, De Nucci G and Cillari E (1995) Prostaglandin E2 regulates inducible nitric oxide synthase in the murine macrophage cell line J774. *Prostaglandins* **49**:105-115.

Mitchell HW, Turner DJ and Noble PB (2004) Cholinergic responsiveness of the individual airway after allergen instillation in sensitised pigs. *Pulm Pharmacol Ther* **17**:81-88.

Mollace V, Muscoli C, Masini E, Cuzzocrea S and Salvemini D (2005) Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacol Rev* **57**:217-252.

Nakano H, Aizawa H, Matsumoto K, Fukuyama S, Inoue H and Hara N (2000) Cyclooxygenase-2 participates in the late phase of airway hyperresponsiveness after ozone exposure in guinea pigs. *Eur J Pharmacol* **403**:267-275.

Nieri P, Daffonchio L, Omini C, Martinotti E and Breschi MC (1992) Changes in airway reactivity to exogenous and endogenous acetylcholine and substance P after anaphylactic bronchoconstriction in anaesthetized guinea-pigs. *J Auton Pharmacol* **12**:403-409.

Nijkamp FP, Van Der Linde HJ and Folkerts G (1993) Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro. Role of the epithelium. *Am Rev Respir Dis* **148**:727-734.

Ogden JE and Moore PK (1995) Inhibition of nitric oxide synthase--potential for a novel class of therapeutic agent? *Trends Biotechnol* **13**:70-78.

Oguma T, Asano K, Shiomi T, Fukunaga K, Suzuki Y, Nakamura M, Matsubara H, Sheldon HK, Haley KJ, Lilly CM, Drazen JM and Yamaguchi K (2002) Cyclooxygenase-2 expression during allergic inflammation in guinea-pig lungs. *Am J Respir Crit Care Med* **165**:382-386.

Peebles RS Jr, Hashimoto K, Morrow JD, Dworski R, Collins RD, Hashimoto Y, Christman JW, Kang KH, Jarzecka K, Furlong J, Mitchell DB, Talati M, Graham BS and Sheller JR (2002) Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* **165:**1154-1160.

Prié S, Cadieux A and Sirois P (1990) Removal of guinea pig bronchial and tracheal epithelium potentiates the contractions to leukotrienes and histamine. *Eicosanoids* **3**: 29-37.

Riendeau D, Percival MD, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Falgueyret JP, Ford-Hutchinson AW, Gordon R, Greig G, Gresser M, Guay J, Kargman S, Leger S, Mancini JA, O'neill G, Ouellet M, Rodger IW, Therien M, Wang Z, Webb JK, Wong E, Xu L,

Young RN, Zamboni R, Prasit P and Chan CC (1997) Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. *Br J Pharmacol* **121**:105-117.

Sadeghi-Hashjin G, Folkerts G, Henricks PA, Verheyen AK, Van Der Linde HJ, Vanark I, Coene A and Nijkamp FP (1996) Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. *Am J Respir Crit Care Med* **153**:1697-1701.

Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG and Needleman P (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA* **90**:7240-7244.

Samb A, Pretolani M, Dinh-Xuan AT, Ouksel H, Callebert J, Lisdero C, Aubier M and Boczkowski J (2001) Decreased pulmonary and tracheal smooth muscle expression and activity of type 1 nitric oxide synthase (nNOS) after ovalbumin immunization and multiple aerosol challenge in guinea pigs. *Am J Respir Crit Care Med* **164**:149-154.

Schildknecht S, Bachschmid M, Baumann A and Ullrich V (2004) COX-2 inhibitors selectively block prostacyclin synthesis in endotoxin-exposed vascular smooth muscle cells. *FASEB J* **18**:757-759.

Schlemper V, Medeiros R, Ferriera J, Campos MM and Calixto JB (2005) Mechanisms underlying the relaxation response induced by bradykinin in the epithelium-intact guinea-pig trachea in vitro. *Br J Pharmacol* **145**:740-750.

Simmons DL, Botting RM and Hla T (2004) Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* **56**:387-437.

Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JJ, Masferrer JL, Seibert K and Isakson PC (1998) Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc Natl Acad Sci USA* **95**:13313-13318.

Stadler J, Harbrecht BG, Di Silvio M, Curran RD, Jordan ML, Simmons RL and Billiar TR (1993) Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J Leukoc Biol* **53**:165-172.

Tunçtan B, Weigl Y, Dotan A, Peleg L, Zengil H, Ashkenazi I and Abacioglu N (2002) Circadian variation of nitric oxide synthase activity in mouse tissue. *Chronobiol Int* **19**:393-404.

Virdis A, Colucci R, Fornai M, Blandizzi C, Duranti E, Pinto S, Bernardini N, Segnani C, Antonioli L, Taddei S, Salvetti A, Del Tacca M. (2005) Cyclooxygenase-2 inhibition improves vascular endothelial dysfunction in a rat model of endotoxic shock: role of inducible nitric-oxide synthase and oxidative stress. *J Pharmacol Exp Ther* **312**:945-953.

FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1: Immunohistochemical detection of COX-1 (A, B) and COX-2 (C, D) performed on serial sections of trachea from normal (A, C, E) and OVA-sensitized (B, D, F) guinea-pigs. (A) Normal trachea shows intense COX-1 staining along the apical surface of columnar epithelial cells with weak cytoplasmic immunoreaction. (B) The thickened pseudostratified columnar epithelium from sensitized animals expresses COX-1 staining at cytoplasmic level, with different intensity degree, and along the ciliar layer that appears widely damaged. (C) COX-2 is present in the cytoplasm of columnar epithelial cells, connective cells of lamina propria (arrows) and condrocytes (c) from normal trachea. (D) Sensitized trachea displays intense COX-2 staining in epithelial cells, mainly of the basal layer, mononuclear cells (arrows) and vessels (arrowheads) of lamina propria, and condrocytes (c). (E, F) No specific immunoreaction is observed in adjacent sections of specimens from normal (E) or sensitized (F) trachea incubated with rabbit preimmune serum. Scale bar = 100 μm.

Fig. 2: COX-1 (A, B) and COX-2 (C, D) immunohistochemical detection in smooth muscle (m) and cartilage (c) performed on serial sections of normal (A, C, E) and OVA-sensitized (B, D, F) guinea-pig trachea. (A) No COX-1 staining is found in tracheal smooth muscle and cartilage from normal animals. (B) No significant amounts of COX-1 staining are detected in smooth muscle and cartilage from sensitized trachea. (C) Low amounts of COX-2 are expressed in condrocytes and smooth muscle cells of normal trachea. (D) Strong COX-2 immunoreaction is found in the cytoplasm of smooth muscle cells and condrocytes of tracheal cartilage from sensitized animals. (E, F). No immunoreaction is observed in muscle and cartilage tissues of normal (E) or sensitized (F) trachea incubated with rabbit preimmune serum. Scale bar = 100 μm.

Fig. 3: Concentration-response curves to histamine obtained in tracheal preparations isolated from normal guinea-pigs, in the absence (closed square) or in the presence of SC-560 10^{-6} M (A) (open circle), DFU 10^{-6} M (B) (open square) or SC-560 10^{-6} M + DFU 10^{-6} M (C) (open diamond). All

responses are reported as percent of contraction induced with 40 mM KCl. Each point represents the mean±S.E. obtained from 6-8 experiments. *P<0.05 by Student t test for unpaired data.

Fig. 4: Concentration-response curves to histamine obtained in tracheal preparations isolated from normal guinea-pigs in the presence of vehicle (control, closed square), or incubated with L-NAME (10^{-4} M) in the absence (closed circle) or in the presence of SC-560 10^{-6} M (A) (open circle) or DFU 10^{-6} M (B) (open square). All responses are reported as percent of contraction induced by 40 mM KCl. Each point represents the mean±S.E. from 6-8 experiments. * P<0.05 by ANOVA and Bonferroni post test.

Fig. 5: Concentration-response curves to histamine obtained in tracheal preparations isolated from OVA-sensitized guinea-pigs, in the absence (closed square) or in the presence of SC-560 10^{-6} M (open circle) (A) or DFU 10^{-6} M (open square) (B), or incubated with L-NAME (10^{-4} M) in the absence (closed circle) or in the presence of SC-560 10^{-6} M (C) (open circle) or DFU 10^{-6} M (D) (open square). All responses are reported as percent of contraction induced by 40 mM KCl. Each point represents the mean±S.E. from 6-8 experiments. *P< 0.05 by Student t test for unpaired data (B) or by ANOVA and Bonferroni post test (D).

Fig. 6: Concentration-response curves to histamine obtained in tracheal preparations isolated from OVA-challenged guinea-pigs, in the absence (closed square) or in the presence of SC-560 10^{-6} M (open circle) or DFU 10^{-6} M (open square), in the absence (A) or in the presence (B) of L-NAME (10^{-4} M), compared to control (closed square) and to L-NAME alone (closed circle). All responses are reported as percent of contraction induced by 40 mM KCl. Each point represents the mean±S.E. from 6-8 experiments. *P<0.05 by ANOVA and Bonferroni post test

Fig. 7: Concentration-response curves to histamine, in the absence (closed square) or in the presence of DFU 10^{-6} M (open square) obtained in tracheal preparations isolated from normal (A) or OVA-sensitized guinea-pigs (B) and deprived of epithelium. All responses are reported as percent of contraction induced by 40 mM KCl. Each point represents the mean±S.E. from 5 experiments. *P<0.05 by Student t test for unpaired data.

Fig. 8: Schematic drawing which shows the proposed mechanism of interaction among COX isoforms and NOS pathways in the modulation of airway contractility in guinea-pig trachea. (A) In normal guinea-pigs, both COX-1 and COX-2 are constitutively expressed at epithelial level with COX-1 at the luminal surface and COX-2 in the cytoplasm. No COX-1 and low amounts of COX-2 are expressed at subepithelial level. Epithelial COX-2 exerts an inhibitory influence on smooth muscle responsiveness. NOS may inhibit COX-2, but this modulatory function is counteracted by epithelial COX-1 which maintains an inhibitory control on NOS activity. The enhancing contractile effect observed with SC-560 may result from the removal of inhibitory control of COX-1 on NOS, with subsequent NOS activation and onset of NO-mediated inhibition on epithelial COX-2, leading to a reduced production of relaxant prostanoids by this COX isoform. B) In ovalbumin-sensitized guinea-pigs, COX-1 is weakly expressed in epithelial cells, while COX-2 is intensely represented both in epithelium and subepithelial cells (inflammatory cells, lamina propria, smooth muscle cells and condrocytes). In this setting, the epithelial COX-1/NOS/COX-2 network is impaired and the contractile responsiveness to histamine is subjected to modulation by COX-2-derived prostanoids produced at subepithelial sites.

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Table 1. Quantitative evaluation of COX-1 and COX-2 expression in tracheae from normal (N) and OVA-sensitized (S) guinea pigs. Immunoreactivity was ranked as – (absent), + (weak), ++ (medium), +++ (strong). Each evaluation derives from 6 experiments.

	EPITHELIUM	LAMINA PROPRIA[a]	CONDROCYTES	SMOOTH MUSCLE
COX-1 N	++[b]	-	-	-
COX-1 S	+[c]	-	-	-
COX-2 N	++	++	+	+
COX-2 S	+++	+++	++	++

[a] subepithelial resident inflammatory cells

[b] immunoreactivity is mainly localized at the cilia of epithelium

[c] immunostaining is weak, except for scattered cells more intensely immunoreactive

Table 2: Effects of SC-560 (10^{-6} M) and DFU (10^{-6} M) on PGE_2 and PGI_2 release from tracheal preparations of normal and OVA-sensitized guinea-pigs, following incubation with histamine (10^{-4} M).

COX-inhibitors were applied 30 min before histamine and supernatants were collected 20 min after incubation with histamine. Data are presented as mean values ± S.E. of 6 experiments.

TREATMENT	***PGE_2 (pg/mg tissue)***		***PGI_2 (pg/mg tissue)***	
	normal	***OVA-sensitized***	***normal***	***OVA-sensitized***
Basal	12.79±4.28	9.43±1.72	20.88±3.21	45.39±2.89[b]
Histamine	35.46±6.02*	14.07±3.48*[b]	40.07±3.68*	71.76±4.92*[b]
Histamine + SC 560	18.38±1.98[a]	7.20±1.30[ab]	27.93±3.98[a]	52.78±3.41[ab]
Histamine + DFU	28.51±5.17*	15.41±3.64*[b]	24.87±3.80[a]	38.79±5.00[ab]
Histamine + SC 560 + DFU	19.08±3.25[a]	6.72±0.74[ab]	17.67±4.15[a]	21.86±3.53*[a]

*P<0.05 vs basal; [a]P<0.05 vs histamine alone; [b]P<0.05 vs normal.

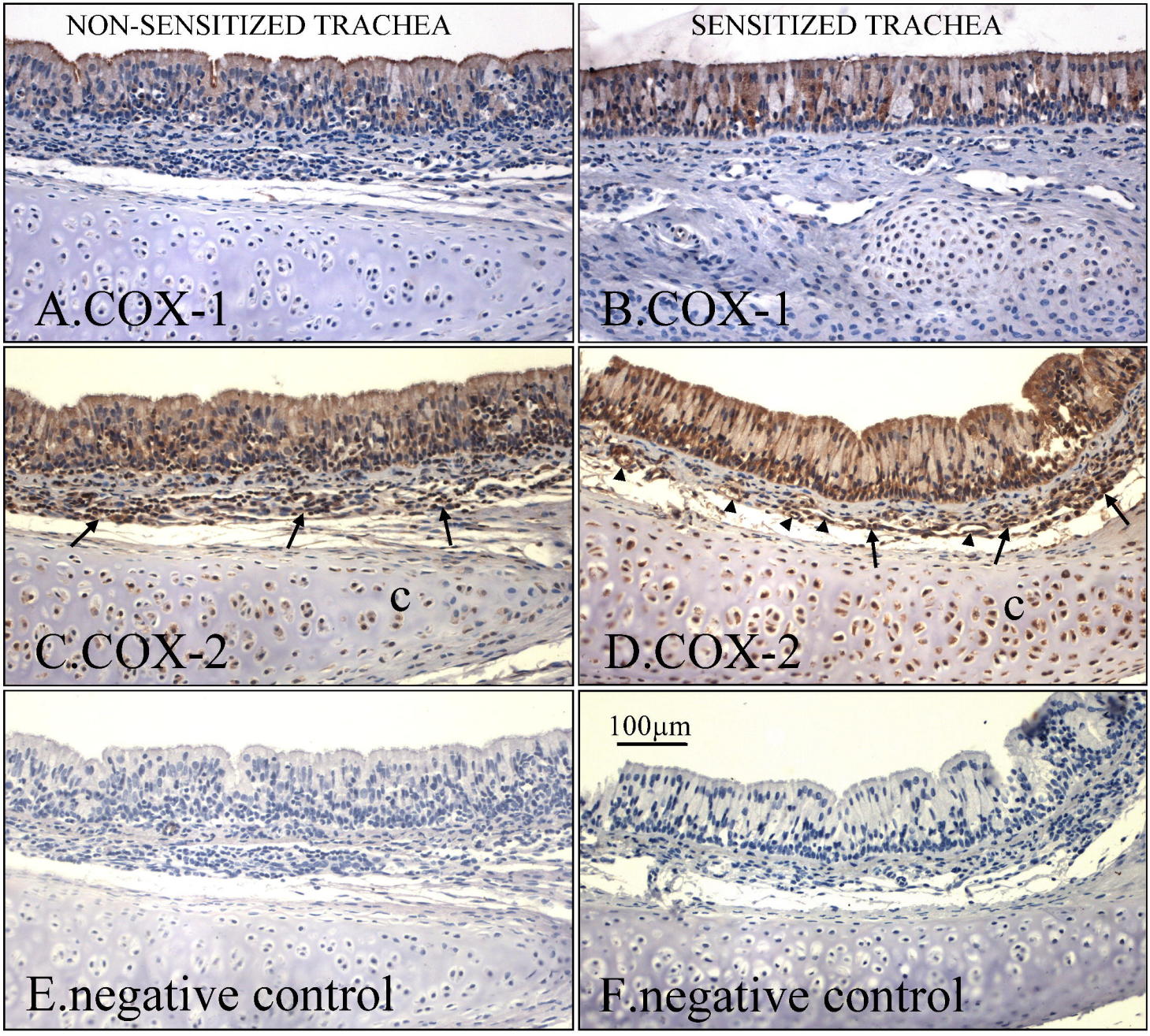
NON-SENSITIZED TRACHEA
SENSITIZED TRACHEA
A.COX-1
B.COX-1
C.COX-2
c
D.COX-2
c
100μm
E.negative control
F.negative control

Figure 1

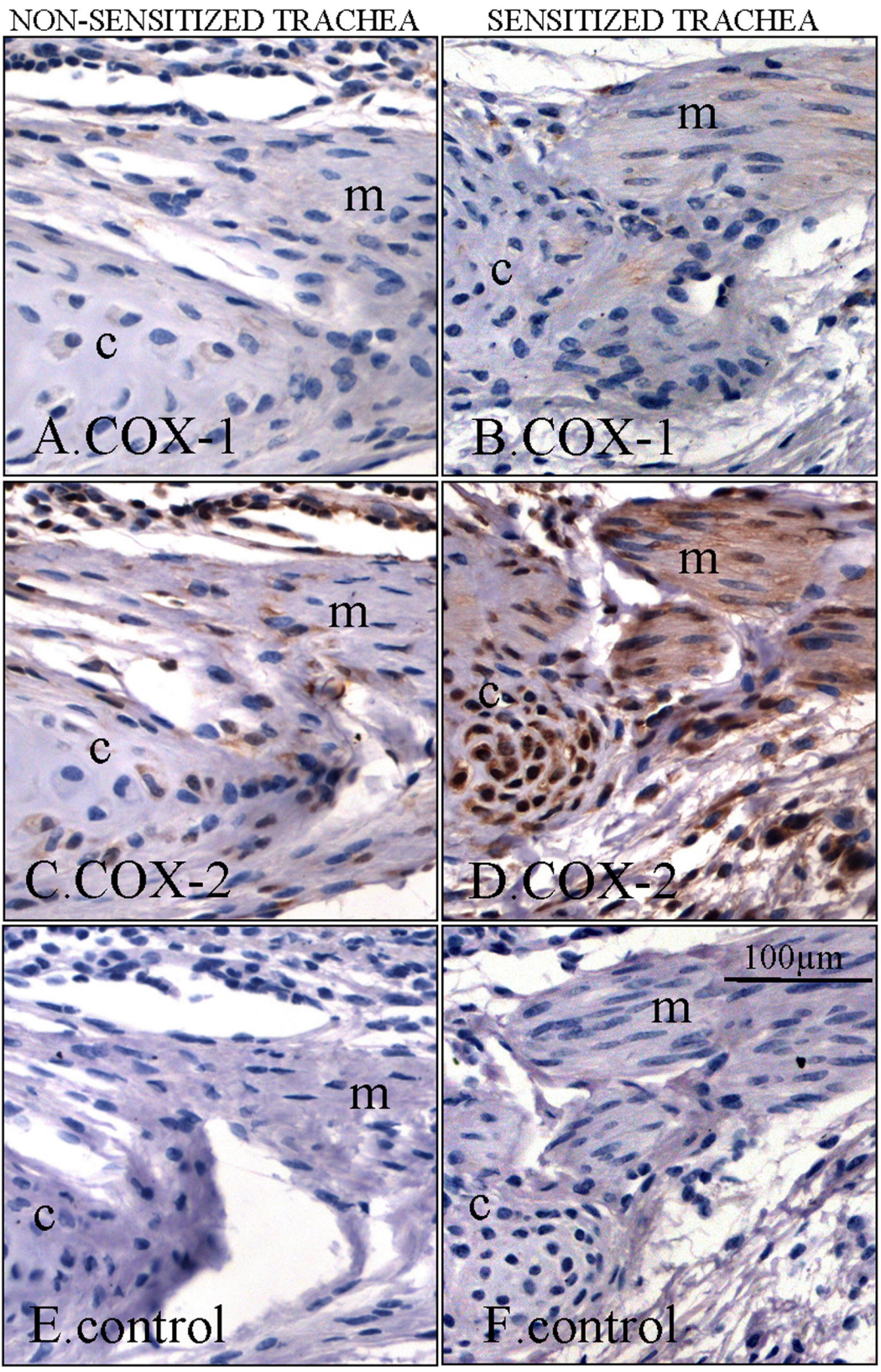
NON-SENSITIZED TRACHEA
SENSITIZED TRACHEA
m
c
A.COX-1
m
c
B.COX-1
m
c
C.COX-2
m
c
D.COX-2
m
c
E.control
100μm
m
c
F.control

Figure 2

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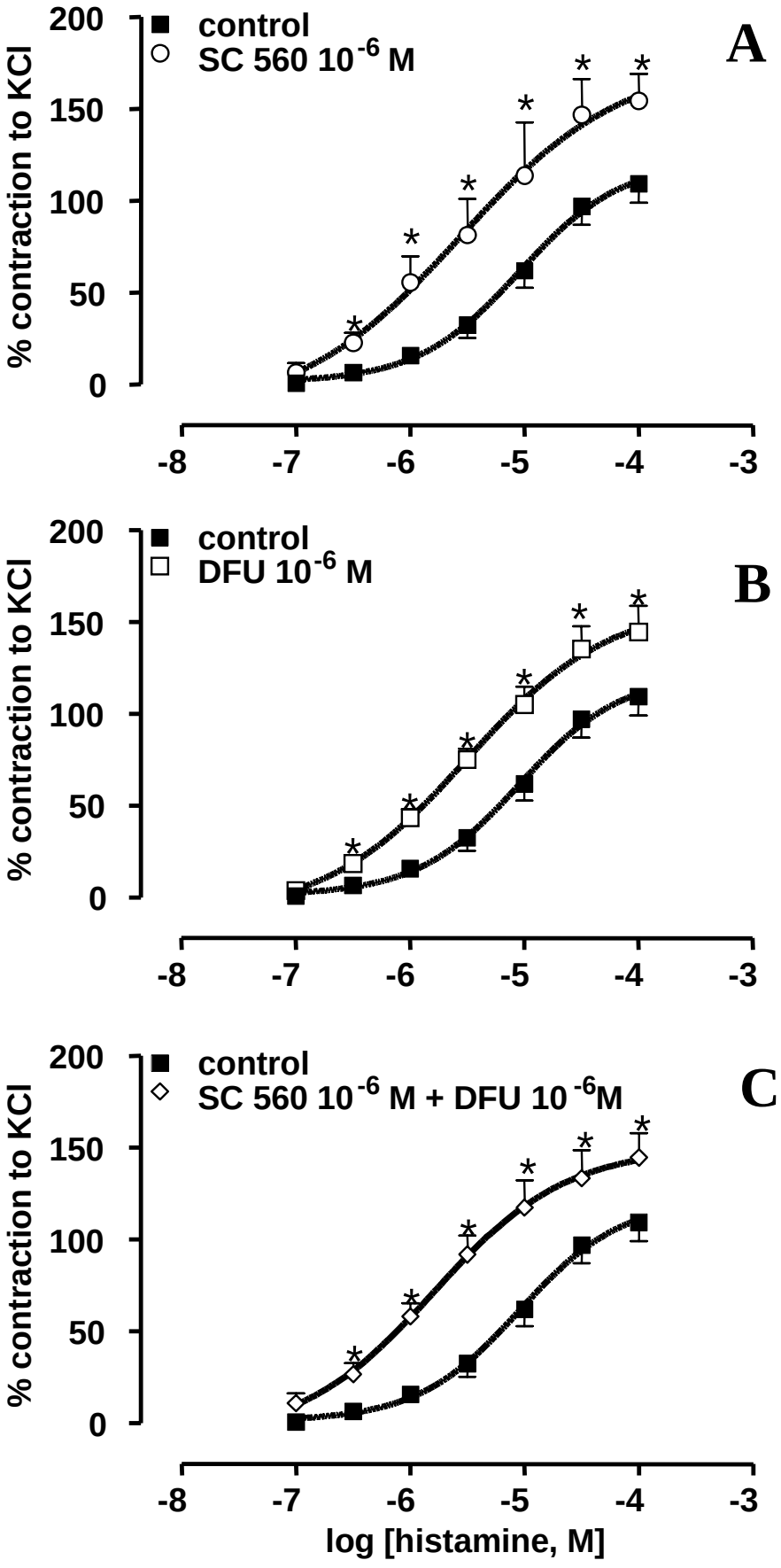

Fig. 3

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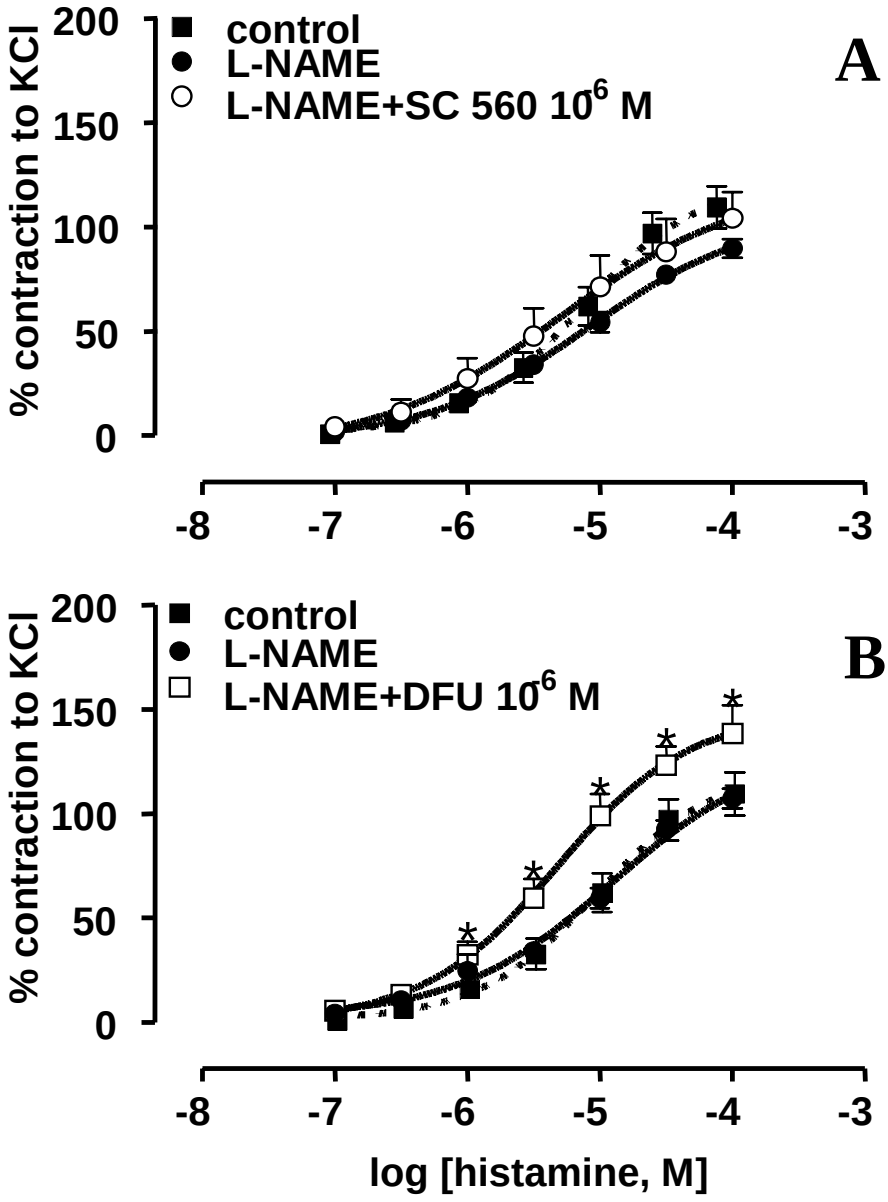

Fig. 4

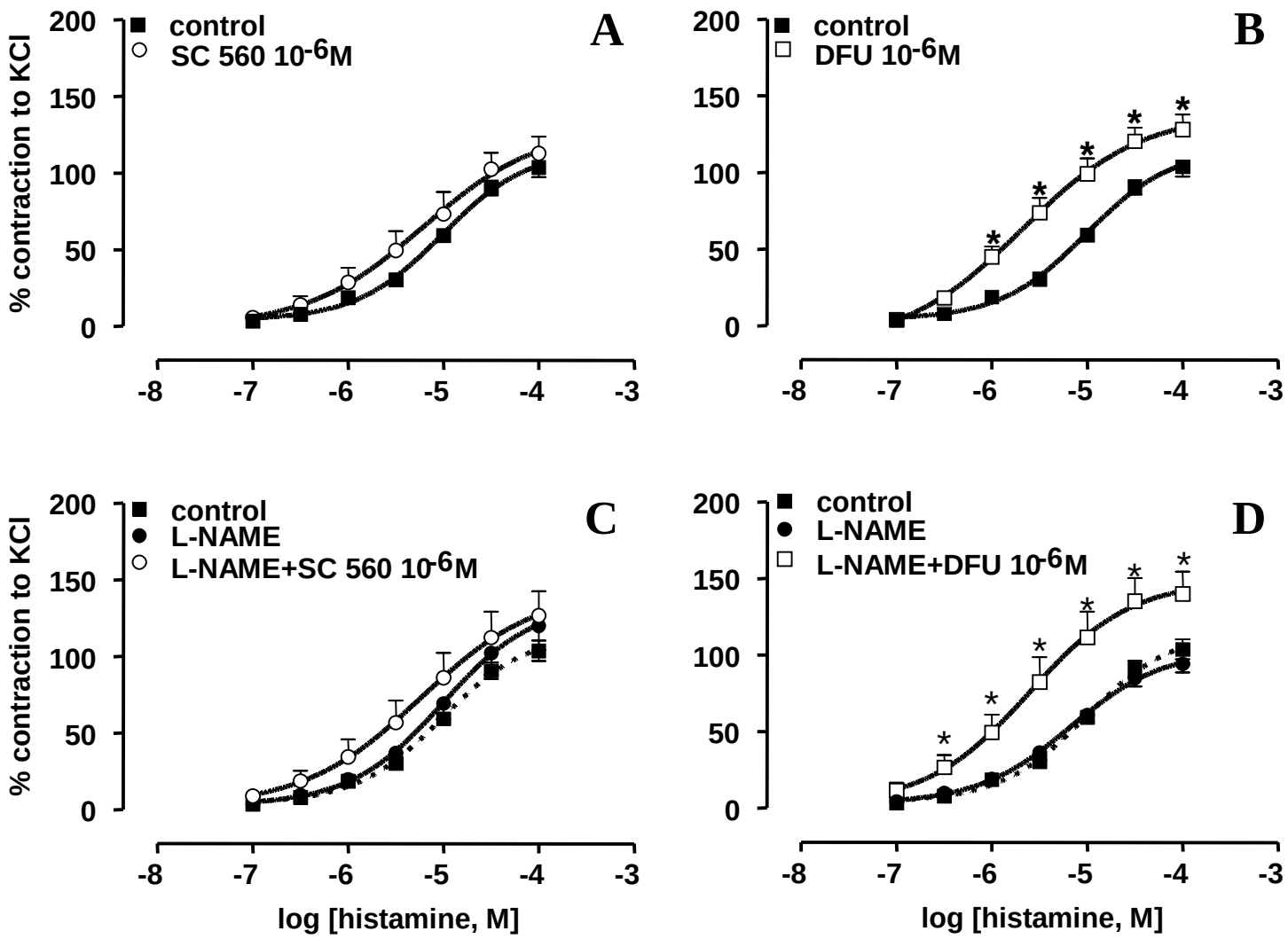

Fig. 5

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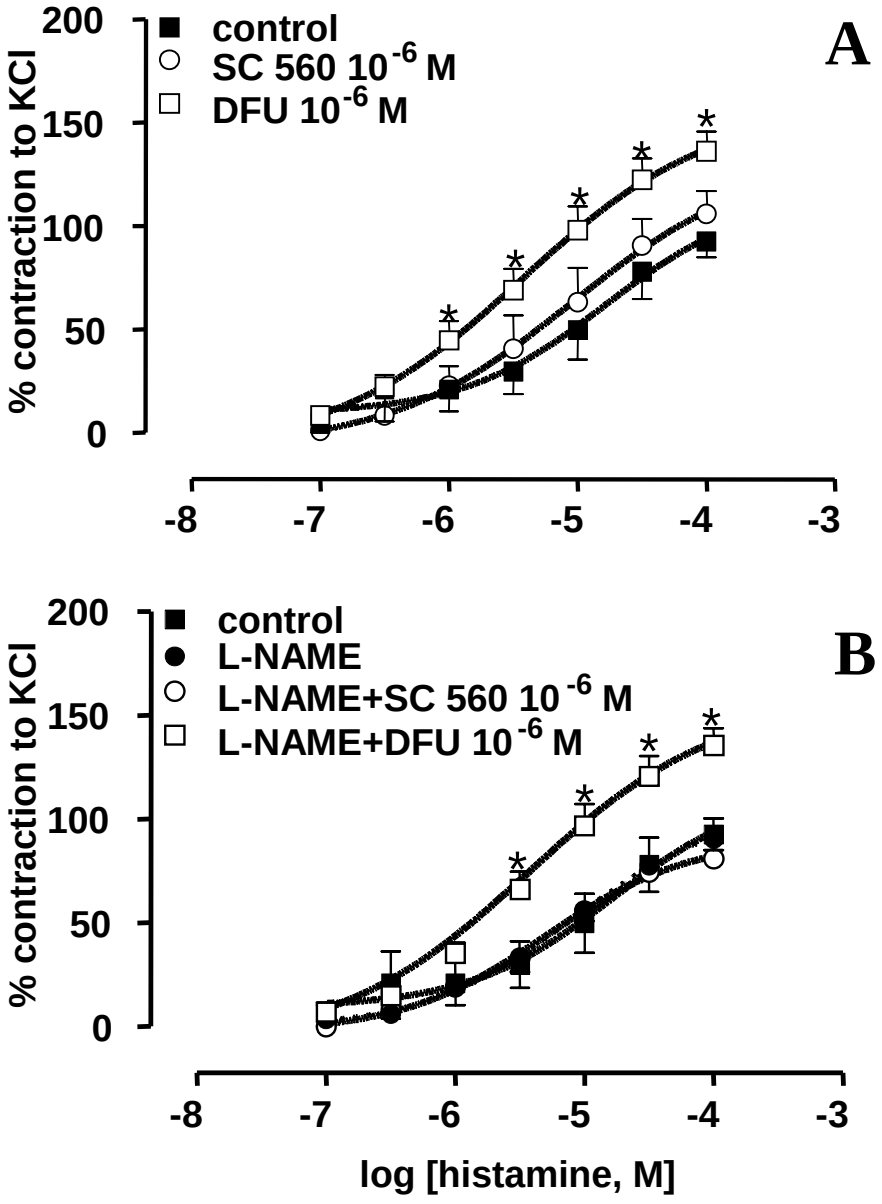

Fig. 6

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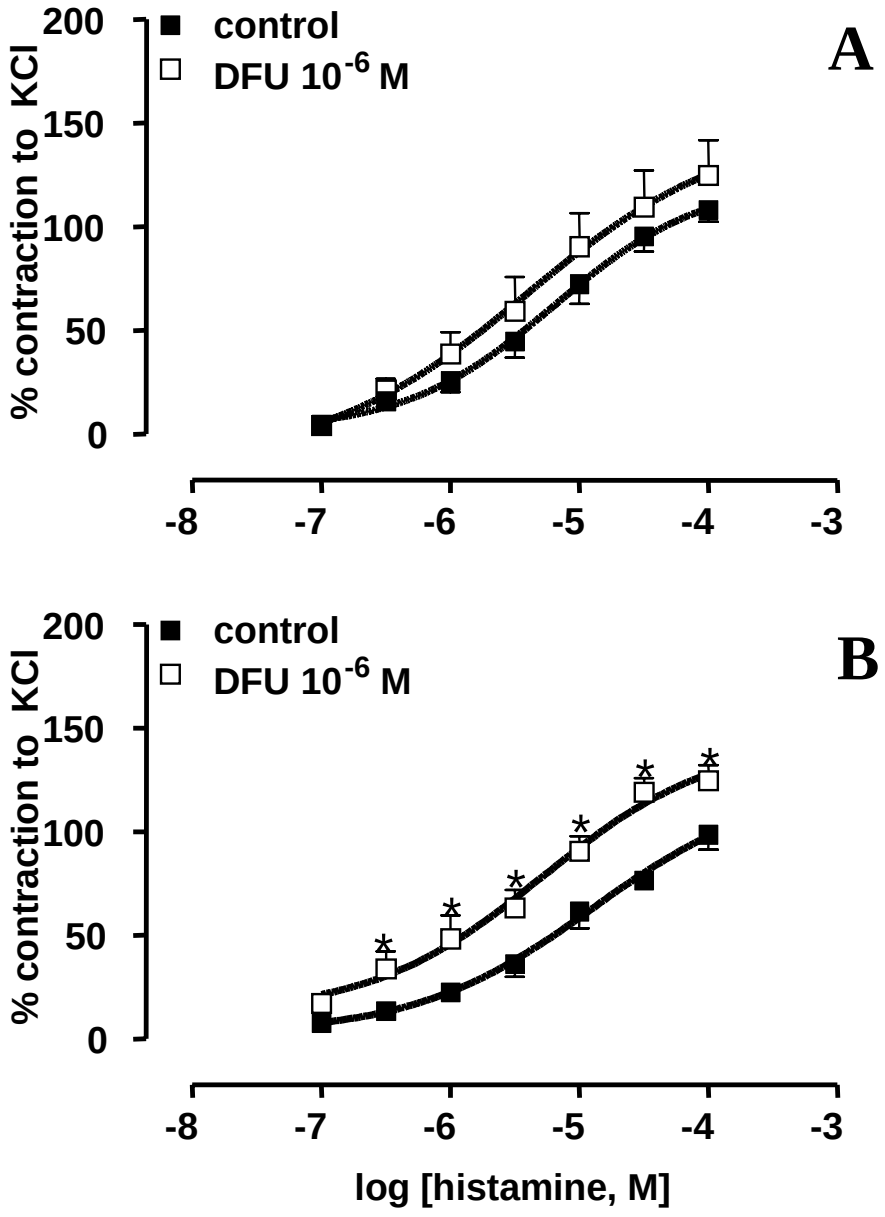

Fig. 7

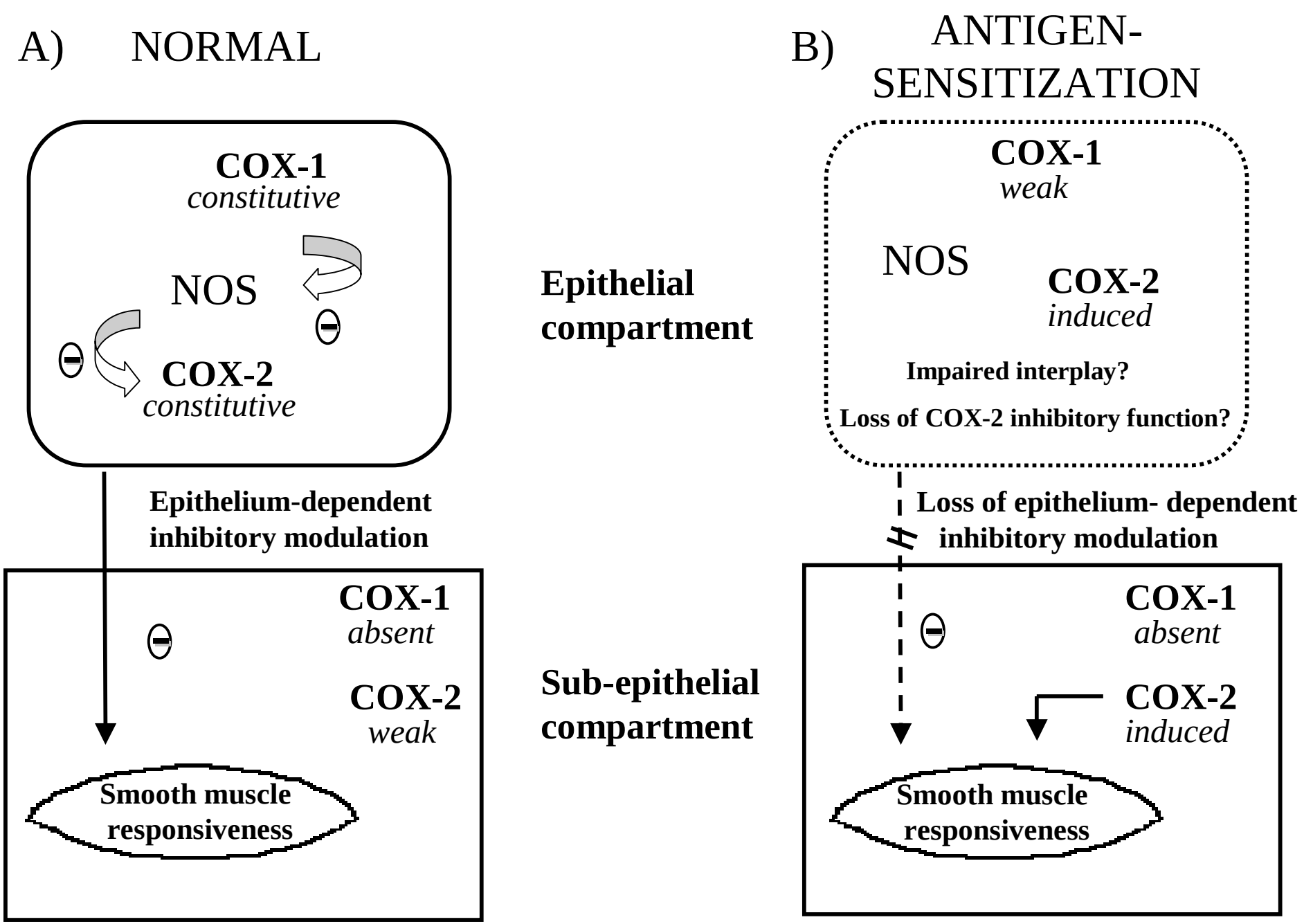

Fig.8