

Reducing agents inhibit the contractile response of isolated guinea-pig main bronchi

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Summary

Background Oxidants are involved in many respiratory disorders, including asthma and chronic obstructive pulmonary diseases. Reduced glutathione (GSH), one of the most important antioxidant compounds against oxidant free radicals, is particularly abundant in the respiratory epithelial lining fluid, where its concentration is increased in inflammatory disorders.

Objective We hypothesized that reducing agents may have a direct effect on airway smooth muscle. Therefore, we studied the effects of GSH on airway smooth muscle contractility in guinea-pig main bronchi. In parallel, we evaluated superoxide anion generation associated with *in vitro* bronchial smooth muscle contraction.

Methods Guinea-pig main bronchi were mounted in organ baths filled with Krebs–Henseleit solution. Concentration–response curves to acetylcholine (Ach) (10^{-9} – 10^{-3} M), carbachol (10^{-9} – 10^{-4} M), or histamine (10^{-9} – 10^{-3} M) were performed in the presence or absence of either reduced or oxidized glutathione (GSSG) (10^{-5} – 10^{-3} M). We also evaluated the effects of GSH and GSSG on allergen-induced contraction in main bronchi obtained from ovalbumin-sensitized guinea-pig. Superoxide dismutase (SOD)-inhibited cytochrome *c* reduction kinetics was performed to evaluate superoxide anion (O_2^-) production during Ach-induced contraction.

Results Reduced but not oxidized glutathione significantly decreased smooth muscle contraction induced by Ach, carbachol, and histamine. Similarly, only the reduced form of glutathione attenuated the bronchoconstriction induced by allergen exposure in bronchi from sensitized animals. Finally, SOD-inhibited cytochrome *c* reduction kinetics demonstrated increased O_2^- production following bronchial smooth muscle contraction. This production was not affected by epithelium removal.

Conclusion Our findings show that GSH decreases bronchial smooth muscle contraction to different stimuli and that oxidant free radicals are produced during bronchial smooth muscle contraction. We suggest that oxidants are involved in the mechanisms of bronchoconstriction and that reducing agents could be a possible therapeutic option for airway obstruction sustained by bronchospasm.

Keywords bronchoconstriction, glutathione, oxidants, reducing agents, smooth muscle

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Introduction

Oxidant generation is part of the normal metabolism of many types of cells, including muscle cells [1, 2]. To protect against exposure to noxious oxidants, the lung has a well-developed antioxidant system [3, 4]. When an imbalance occurs between oxidants and antioxidants, in favour of oxidants, oxidative stress is said to occur. Recent evidence suggests that oxidants play a role in the pathogenesis of many respiratory disorders, including asthma and smoking-related diseases such as chronic obstructive pulmonary diseases (COPD) [5–7]. In

smokers, the oxidant burden in the lung is enhanced both directly from oxidants in cigarette smoke and also by the release of reactive oxygen species from macrophages and neutrophils [3]. Much of the evidence for the involvement of reactive oxygen species in the pathogenesis of asthma is indirect, since there are no specific and reliable methods to assess oxidative stress *in vivo*. However, the inflammation that is characteristic of asthma probably results in increased oxidative stress in the airways, as numerous surrogate markers of oxidative stress have been shown to be elevated in asthmatic subjects [8]. In experimental models, oxidants produce airway bronchoconstriction and/or hyper-responsiveness both *in vitro* and *in vivo* [9–21], while cigarette smoke acutely increases airway resistance via a direct oxidant-mediated mechanism [22].

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Reduced glutathione (GSH) is one of the most important reducing agents against free radicals and other oxidants [23, 24]. It is present in each cell in millimolar concentration [25] and also in the extracellular compartment, although at lower concentrations. GSH is particularly abundant in the respiratory epithelial lining fluid (ELF), where its concentration exceeds plasma levels by approximately 100-fold [25, 26]. The concentration of GSH is increased in the ELF of smokers and of patients with respiratory inflammatory disorders [25, 26].

Although the relaxing effect of reducing agents has been studied in experimental models [27–29], and their capability to prevent airway hyper-responsiveness has been investigated both *in vitro* and *in vivo* [30, 31], little is known about the influence of the redox state on smooth muscle contraction mechanisms [18]. In particular, the direct effect of reducing agents on normal bronchial smooth muscle contractile response, i.e. in the absence of any induced hyper-reactivity, has never been studied before. Moreover, the mechanisms underlying the effects of redox state changes on smooth muscle responses have been minimally studied.

Therefore, the aim of our study was to examine the effect of GSH on airway smooth muscle contractile responses in guinea-pig main bronchi. In the search of a possible target for this reducing agent, superoxide anion generation during bronchial smooth muscle contraction was also evaluated.

Materials and Methods

Bronchial ring preparations

This study was approved by the animal experiment review committee of Ferrara University School of Medicine. We anaesthetized male Hartley-outbred guinea-pigs (300–400 g body weight, Morini farm, Reggio Emilia, Italy) with pentobarbital sodium (50 mg/kg i.p.). The lungs were removed rapidly and immersed in oxygenated Krebs–Henseleit solution containing the following (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 D-(+)-glucose. The main bronchi were dissected free of loose connective tissue and then cut into 2–3-mm-thick rings. The rings were mounted in glass chambers filled with Krebs–Henseleit solution that was maintained at 37 °C and aerated continuously by bubbling it with a mixture of 95% O₂ and 5% CO₂, which produced a pH of 7.4. We continuously measured isometric tension in bronchial smooth muscle rings connected to a force-displacement transducer (Grass FTO3) and recorded the responses on a Battaglia Rangoni model KV380 polygraph recorder. Rings were equilibrated at a resting tension of 0.5 g for 90 min and washed with fresh Krebs–Henseleit solution every 20 min.

Effect of glutathione on contraction of guinea-pig main bronchi

Cumulative concentration–response curves to acetylcholine (ACh) (10^{−9}–10^{−3} M), carbachol (10^{−9}–10^{−4} M), or histamine (10^{−9}–10^{−3} M) were obtained in the presence or absence of either reduced glutathione (GSH, 10^{−5}–10^{−3} M, 30 min pre-treatment) or oxidized glutathione (GSSG, 10^{−3} M, 30 min pre-treatment).

Effect of glutathione on ovalbumin (OVA)-induced contraction in sensitized guinea-pig main bronchi

Young, male, pathogen-free Hartley guinea-pigs (initial weight approximately 350 g) were sensitized to OVA by daily exposure to aerosolized OVA for 5 min, 5 days a week, for 2 weeks. During the exposure, guinea-pigs were placed in a Plexiglas chamber (36 × 24 × 20 cm), which was connected to an ultrasonic nebulizer (Model 100; Devilbiss, Somerset, PA, USA) and placed under a negative-pressure exhaust hood. OVA/saline solution (wt/vol concentration: 1%) was nebulized with an output rate of 0.2 mL/min and a droplet size ranging from 0.5 to 30 µm. A matching group of guinea-pigs was exposed to saline aerosol only as controls. Twenty-four hours after the last exposure, main bronchi were obtained as described above.

In vitro response to 10^{−5} M OVA was evaluated in the presence or absence of either GSH 10^{−4} M or GSSG 10^{−4} M (30 min pre-treatment).

In vitro superoxide anion (O₂[−]) release during guinea-pig main bronchi contraction

O₂[−] generated and released in the medium during guinea-pig main bronchi contraction was evaluated by superoxide dismutase (dioxide 1-dismutase or SOD)-inhibited cytochrome *c* reduction kinetics as previously described [32].

Experimental design Six paired main bronchi were prepared and mounted as described above. When indicated, the epithelium was previously removed by gently rubbing the luminal surface with a gauze according to a standardized protocol [33]. Removal of the epithelium was confirmed histologically [33].

Thirty minutes after equilibration, 1 mL of Krebs–Henseleit medium was collected. The experimental conditions were as follows: (1) resting bronchial rings and (2) bronchial rings exposed to ACh 10^{−4} M 5 min before sampling. In parallel, the same experimental conditions were applied to bronchial rings without epithelium. Aeration bubbling was stopped for the duration of the experiment.

To determine wet weight, the bronchial rings were blotted on filter paper at the end of the experiments and weighed.

Cytochrome *c* reduction kinetics We tested superoxide production by cytochrome *c* reduction kinetics, according to previously published methods [34, 35] in a UviKon 830 spectrophotometer (Kontron Instruments), in 1-mL quartz cuvettes at 37 °C for 20 min. Each cuvette contained 0.8 mL of Krebs–Henseleit medium obtained as described above and 0.2 mL of 10^{−4} M cytochrome *c*, with or without SOD (500 U/mL). The concentration of reduced cytochrome was calculated dividing cytochrome absorbance at 550 nm by the molar extinction coefficient of cytochrome *c* (ϵ = 29500). The difference in absorbance in the presence or absence of SOD was obtained after 5 min of kinetics, when the kinetic slope of cytochrome *c* reduction was steepest.

The assay is based on the ability of SOD to catalyse the dismutation of superoxide to hydrogen peroxide and molecular oxygen; thus, the portion of cytochrome *c* reduction kinetics that is inhibited by SOD is specifically caused by superoxide production. Since one molecule of

superoxide reduces one molecule of cytochrome *c*, the total amount of reduced cytochrome corresponds to the total amount of superoxide produced, so allowing a stoichiometric measure of superoxide generation.

Data were normalized per milligram of tissue wet weight.

In order to evaluate whether Ach affected cytochrome *c* reduction kinetics, we performed this assay in six samples of medium alone and in six samples of medium containing 10^{-4} M Ach.

Data analysis

Contractions were expressed as a percentage of the active tension obtained in response to 10^{-3} M Ach. The shift of the concentration–response curve was evaluated by calculating the concentration eliciting 50% of the maximum response (EC_{50}), an index of the smooth muscle sensitivity. Data are given as mean $\pm$ SEM, except for the EC_{50} , which is expressed as geometric mean and geometric standard error of the mean. Concentration–response curves were compared by two-way ANOVA. Groups were compared by a two-tailed *t*-test to assess the statistical significance of the results at each concentration. Micromoles of O_2^- /mg tissue wet weight were expressed as mean $\pm$ SEM. The two groups were compared by a two-tailed *t*-test. Values of $P < 0.05$ were considered significant.

Drugs and chemicals

Ach, carbamylcholine chloride, histamine, albumin egg (grade VI), SOD, cytochrome *c*, GSH, and GSSG were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Ach, carbachol, and histamine were diluted in distilled water. Stock solutions were made at concentrations 1000-fold greater than the final bath concentrations, to minimize changes in isolated organ bath volume, pH, and osmolarity.

Results

Effect of glutathione on bronchial smooth muscle response

Ach caused a concentration-dependent isometric contraction of bronchial smooth muscle that was significantly decreased by GSH 10^{-3} and 10^{-4} M ($P < 0.05$ by ANOVA). Reduced responses were observed mainly at lower concentrations, whereas no difference was found in terms of maximal response (Fig. 1a and b). EC_{50} values for Ach were significantly increased in bronchial rings pre-treated with GSH 10^{-3} M [5×10^{-7} (1.39) M vs. 3.5×10^{-6} (1.31) M in control and pre-treated bronchial rings, respectively; $P < 0.05$] and 10^{-4} M [4.58×10^{-7} (1.4) M vs. 1.08×10^{-6} (1.51) M in control and pre-treated bronchial rings, respectively; $P < 0.05$].

Bronchial smooth muscle responses to lower Ach concentrations (10^{-9} – 10^{-6} M) were significantly reduced also by GSH 10^{-5} M, although both the concentration–response curves to Ach, by ANOVA, and the EC_{50} values were not significantly affected by GSH 10^{-5} M (data not shown). Pre-treatment with GSSG (10^{-4} – 10^{-3} M, Fig. 1c) did not modify the responses to Ach.

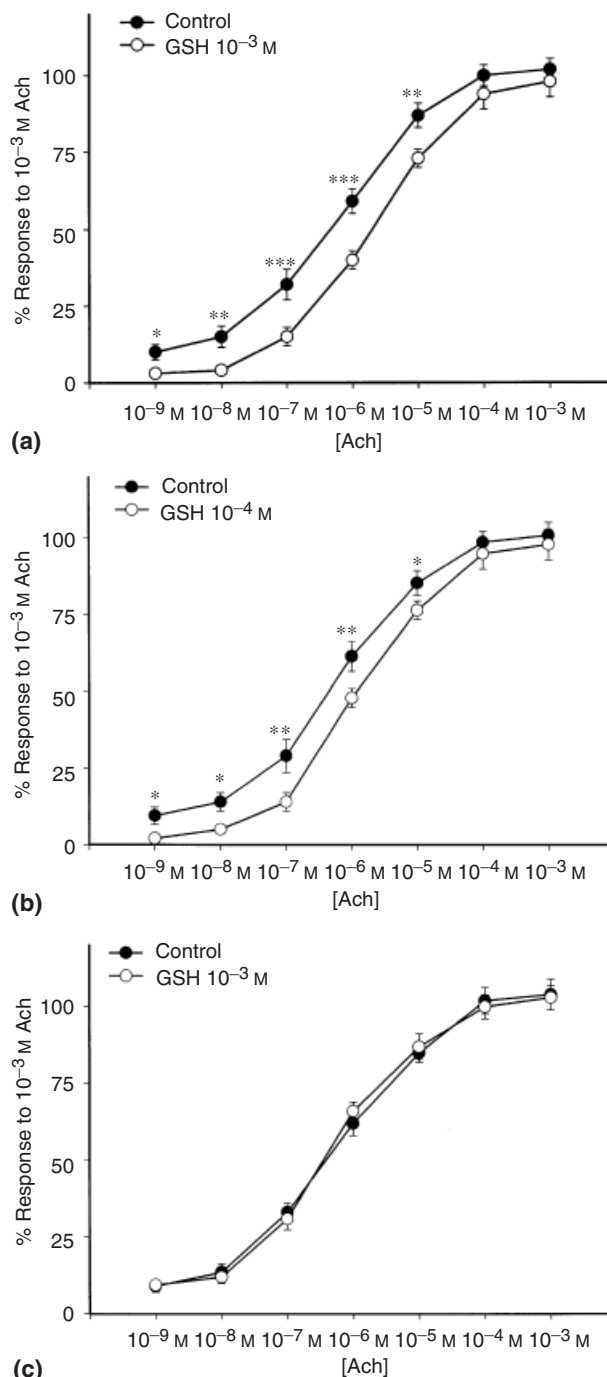


Fig. 1. Dose–response curves to acetylcholine (Ach) in guinea-pig main bronchi in the presence (open circles) or absence (closed circles) of 10^{-3} M reduced glutathione (a), 10^{-4} M reduced glutathione (b), or 10^{-3} M oxidized glutathione (c). * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control. (a) Each point is the mean $\pm$ SEM of nine experiments. (b) Each point is the mean $\pm$ SEM of eight experiments. (c) Each point is the mean $\pm$ SEM of 10 experiments. GSH – reduced glutathione; GSSG – oxidized glutathione.

GSH 10^{-4} M significantly reduced isometric responses to carbachol ($P < 0.05$ by ANOVA, Fig. 2) and increased the EC_{50} values for carbachol [3.38×10^{-7} (1.09) M vs. 8.7×10^{-7} (1.13) M in control and pre-treated bronchial rings, respectively; $P < 0.05$], whereas GSSG 10^{-4} M did not affect carbachol dose–response curves (data not shown).

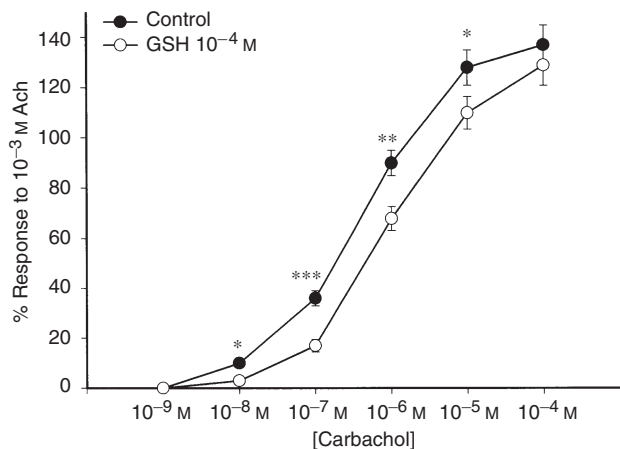


Fig. 2. Dose-response curves to carbachol in guinea-pig main bronchi in the presence (open circles) or absence (closed circles) of 10^{-4} M reduced glutathione. Each point is the mean $\pm$ SEM of six experiments. * P < 0.05 vs. control; ** P < 0.01 vs. control; *** P < 0.001 vs. control. GSH – reduced glutathione; Ach – acetylcholine.

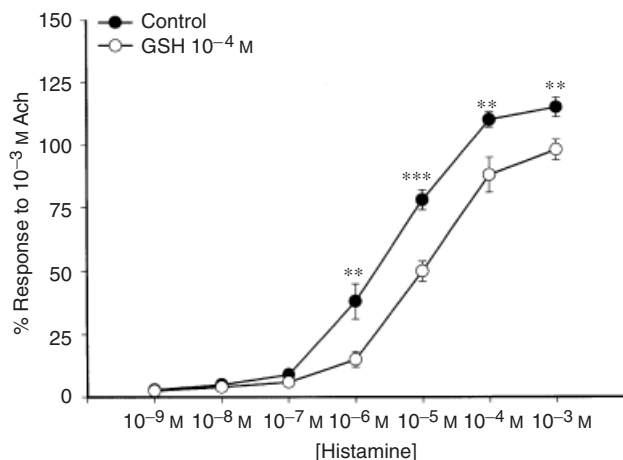


Fig. 3. Dose-response curves to histamine in guinea-pig main bronchi in the presence (open circles) or absence (closed circles) of 10^{-4} M reduced glutathione. Each point is the mean $\pm$ SEM of five experiments. ** P < 0.01 vs. control; *** P < 0.001 vs. control. GSH – reduced glutathione; Ach – acetylcholine.

Force generation induced by histamine was reduced (P < 0.05 by ANOVA; Fig. 3), and EC_{50} values for histamine were increased [3.06×10^{-6} (1.22) M vs. 10^{-5} (1.24) M in control and pre-treated bronchial rings, respectively; P < 0.05] by GSH 10^{-4} M, but not by GSSG 10^{-4} M (data not shown).

Also, the contractile responses to OVA were significantly reduced by the reduced but not the oxidized form of glutathione (10^{-4} M, Fig. 4).

O₂⁻ generation by Ach-induced contraction in guinea-pig bronchi

O_2^- was virtually absent in the medium when bronchi were in resting conditions. Conversely, O_2^- was released in the medium when bronchi were exposed to Ach 10^{-4} M (0.18 ± 0.07 μ M/mg vs. 1.28 ± 0.17 μ M/mg in resting conditions and after Ach, respectively, P < 0.01; Fig. 5).

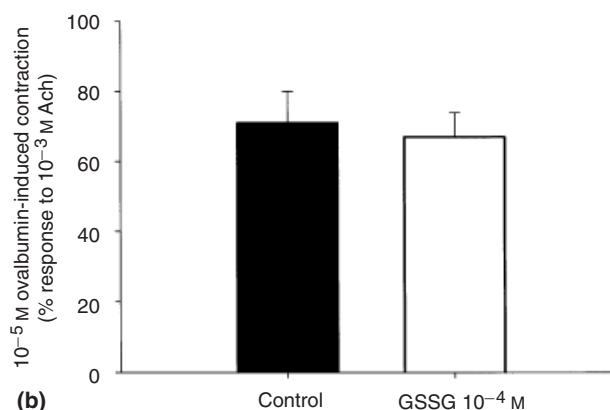
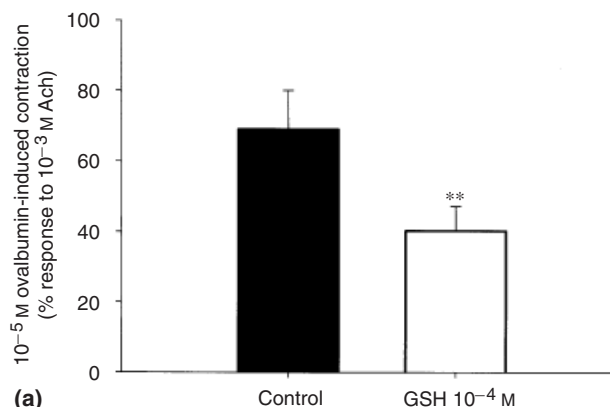


Fig. 4. Contractile response to 10^{-5} M ovalbumin (OVA) of main bronchi from sensitized guinea-pig in the presence (empty) or absence (filled) of 10^{-4} M reduced (a) or oxidized (b) glutathione. Each column is the mean $\pm$ SEM of six experiments. GSH – reduced glutathione; GSSG – oxidized glutathione; ** P < 0.01 vs. control.

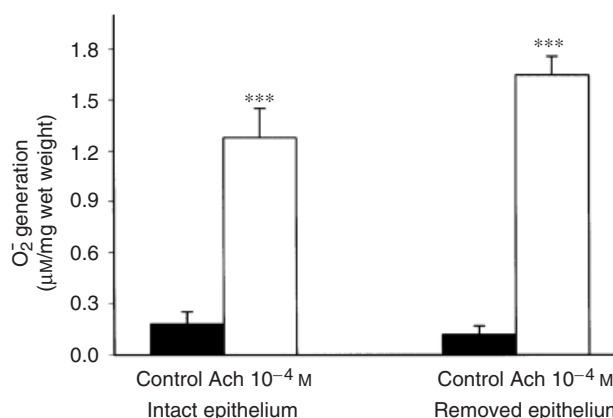


Fig. 5. Superoxide anion (O_2^-) released in the medium by guinea-pig bronchi, with or without epithelium, in resting conditions (control) and after exposure to 10^{-4} M Ach. Ach – acetylcholine. *** P < 0.001 vs. control.

O_2^- production was not affected by epithelial removal (0.12 ± 0.05 μ M/mg vs. 1.65 ± 0.11 μ M/mg in resting conditions and after Ach, respectively, P < 0.01; Fig. 5). Histological analysis confirmed that $94.8 \pm 1.5\%$ of the epithelium had been removed. Production of O_2^- was not detected in the presence of medium alone, with or without Ach 10^{-4} M ($n = 6$).

Discussion

In this study we have demonstrated that the reducing agent GSH affects *in vitro* bronchial smooth muscle response to different pharmacological agonists and that contraction of bronchial rings is associated with superoxide anion production.

Several studies showed that oxidant free radicals produce smooth muscle contraction and/or hyper-reactivity both *in vivo* and *in vitro* [9–21, 36]. Although the capability of antioxidants to protect against experimentally induced airway hyper-responsiveness and to induce smooth muscle relaxation has been demonstrated both *in vivo* and *in vitro* [9, 21, 27–31, 37], the direct effect of antioxidants on airway smooth muscle contractility has been minimally studied. We now report for the first time that pre-treatment with the reducing agent GSH has a direct inhibitory action on guinea-pig bronchial smooth muscle contractile response in 'basal condition', *i.e.* in bronchial airways neither pre-contracted nor hyper-responsive. Our finding that reduced but not oxidized glutathione decreases bronchial contractility demonstrates that the effect of GSH is not related to its chemical structure, but to its ability to affect redox state. Therefore, it is conceivable that GSH directly affects the contractile response through its antioxidant activity. In contrast, it remains to be elucidated whether the GSH antioxidant action is responsible for the relaxant effect this compound exerts on smooth muscle. Indeed, a similar vasodilator effect was produced by GSH and GSSG on coronary arteries [27]. Moreover, a release of NO has been shown to be involved in GSH-induced relaxation both in vascular and in bronchial smooth muscle [27, 29]. It seems therefore likely that the GSH effect on the contractile phase of the airway smooth muscle response is attained through mechanisms that are different from those involved in its relaxing effect.

Antioxidants could potentially affect airway smooth muscle response through several mechanisms. Since oxygen radicals have been reported to affect airway smooth muscle response [9–15], we tested the hypothesis that oxidant free radicals are produced during airway smooth muscle contraction. We found that O_2^- generation occurs in guinea-pig bronchi during Ach-induced contraction, indicating that oxidant generation is clearly associated with bronchial smooth muscle contraction. Therefore, the decreased bronchial response to contractile agonists determined by GSH may be explained by the effect of this reducing agent on the oxidant burst occurring during smooth muscle contraction.

Interestingly, in a study conducted in a different model, cat isolated airways, O_2^- -generating systems did not produce significant effects on the contractile response, but determined a sustained contraction in the presence of SOD, indicating the conversion of O_2^- to H_2O_2 as responsible for the increase in smooth muscle contraction [38]. The same oxidative pathway that leads to muscle contraction could be involved in our experimental conditions. Indeed, since GSH has a scavenger activity both on O_2^- and on H_2O_2 [39], it is possible that GSH reduces airway smooth muscle contraction, at least partially, by decreasing H_2O_2 production.

To investigate whether epithelial cells are involved in the production of O_2^- observed in bronchial rings exposed to Ach, we evaluated the effect of epithelium removal in our experimental setting. In the absence of epithelial cells, we

observed a trend toward ($P = 0.09$) increased O_2^- production after Ach, which parallels a similar increase in bronchial smooth muscle contraction (data not shown). These data indicate that epithelial cells are not involved in the O_2^- generation occurring during smooth muscle contraction and suggest that superoxide may be directly produced by the airway smooth muscle.

GSH may exert its antioxidant/reducing activity via direct or indirect effects on the intracellular redox state [23]. Indeed, extracellular GSH can be transported across cell membrane or modify intracellular GSH concentrations without any transmembrane transport [40]. Recent studies have documented that glutathione can inhibit Ca^{2+} release from isolated sarcoplasmic reticulum [41–43]. Similarly, it has been demonstrated that the nitric oxide donor *S*-nitrosoglutathione relaxed canine tracheal smooth muscle by decreasing Ca^{2+} sensitivity [44, 45]. Therefore, our observations could also be dependent on the effect of GSH on Ca^{2+} release. Although additional investigation will be needed to clarify this point, we suggest that the reduced airway smooth muscle contractility caused by GSH may originate by a combination of the GSH effects on the amount of oxidant free radical produced during a contractile response and on Ca^{2+} release.

The intracellular GSH homeostasis is critical to normal cellular physiological processes and represents one of the most important antioxidant defence systems in the lung [24]. An imbalance of oxidant/antioxidant in favour of oxidants contributes to the pathogenesis of several inflammatory lung diseases [46, 47]. Indeed, oxidant stress is associated with airway inflammation both in asthma and COPD. Our results show that airway smooth muscle responsiveness can be reduced by reducing agents, suggesting that to restore a balance in the redox state of the airway tissue could have a positive effect on the increased responsiveness associated with airway inflammation. It is important to underline that the concentrations of GSH utilized in the present study are those present in the fluid that line respiratory epithelial cells [32]. The magnitude of the inhibition exerted by glutathione on smooth muscle contractile response, evaluated as EC_{50} shift, ranged from 0.5 to 0.85 log concentration of the agonist. This is a pharmacologically relevant effect in the *in vitro* system we adopted [48] and may well result in a significant protection against bronchospastic agents *in vivo*.

In conclusion, our results indicate reducing agents as possible therapeutic options for respiratory diseases characterized by airway obstruction linked to bronchial smooth muscle contraction.

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