

Nebulized Glutathione Induces Bronchoconstriction in Patients with Mild Asthma

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To assess the effects on bronchial responsiveness of nebulized glutathione (GSH), one of the most efficient scavengers of oxidant substances in the airways, we studied eight patients with mild asthma (FEV_1 , $88 \pm 11\%$ predicted [SD]) in a randomized, double-blind, cross-over, placebo-controlled fashion. Bronchial challenge was measured using both FEV_1 and total pulmonary resistance (Rrs) by the forced oscillation technique. Patients received nebulized GSH (600 mg with 4 ml of 0.9% sodium chloride) or placebo (identical saline solution) over a period of 25 min, 1 wk apart. Placebo provoked subclinical mild bronchoconstriction (changes from baseline: FEV_1 , -1% ; Rrs, $+17\%$); by contrast, GSH caused major airway narrowing (changes from baseline: FEV_1 , -19% ; Rrs, $+61\%$) and induced cough (four patients) or breathlessness (three patients). Differences between placebo and GSH after challenge were also noticeable in both FEV_1 ($p = 0.03$) and Rrs ($p = 0.02$). Neither osmolarity ($660 \text{ mosm} \cdot \text{kg}^{-1}$) nor pH (3.0) of the GSH solution accounted for these effects. Nebulized salbutamol (5.0 mg) given before the GSH challenge blocked GSH-induced bronchoconstriction. Furthermore, GSH-induced FEV_1 falls were inversely correlated with metabisulfite bronchoprovocation (provocative dose [PD₂₀], $1.49 \pm 1.83 \mu\text{mol}$) but not with methacholine challenge. The detrimental effects of nebulized GSH on the airway bronchial tone in patients with mild asthma strongly suggests bronchoconstriction provoked by sulfite formation. Marrades RM, Roca J, Barberà JA, de Jover L, MacNee W, Rodriguez-Roisin R. Nebulized glutathione induces bronchoconstriction in patients with mild asthma.

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An imbalance between oxidants and antioxidants may affect patients with obstructive airway disease, such as bronchial asthma and chronic obstructive pulmonary disease (COPD). There are data suggesting that increased production of reactive oxygen species (ROS), or oxidants, by inflammatory cells contributes to the pathogenesis of asthma (1). Glutathione (L- γ -glutamyl-L-cysteinyl-glycine, or GSH), a sulfhydryl-containing tripeptide produced by most mammalian cells, is an efficient scavenger of ROS. Glutathione is present in the epithelial lining fluid (ELF) of the normal lower respiratory tract (2), where it is thought to play a major role in preventing or minimizing the adverse consequences of ROS. Under appropriate conditions, an increase in one or more antioxidants might offset increases in oxidant production. Both systemic and ELF deficiency of

GSH have been demonstrated in patients with idiopathic pulmonary fibrosis (IPF) (3) and cystic fibrosis (4), and in individuals who are HIV-seropositive but symptom-free (5). GSH has been nebulized safely in patients with IPF to augment GSH levels on the respiratory epithelial surface, thus enhancing antioxidant defense and controlling fibroblast proliferation (6). Likewise, in HIV-seropositive individuals, GSH has been shown efficacious, apparently by improving local host defenses (7).

Decreased peripheral blood GSH peroxidase activity has been documented in patients with asthma, thereby suggesting reduced antioxidant defenses and increased susceptibility to ROS (8, 9). Similarly, nocturnal asthma has been related to an imbalance between oxidants and antioxidants in the ELF (10). Smith and co-workers (11) found increased amounts of GSH in bronchoalveolar lavage fluid in patients with stable asthma amounts that were inversely correlated with bronchial hyperresponsiveness. Recently, Vachier and colleagues (12) demonstrated an enhancement of ROS formation by polymorphonuclear neutrophils and monocytes in stable asthmatic patients. Thus, the potential benefits of GSH inhalation in patients with unstable asthma or during an acute severe exacerbation, to reinforce the antioxidant defenses, could enhance the efficacy of both bronchodilators and anti-inflammatory agents. However, many substances given by nebulization may affect the underlying bronchomotor tone, particularly in asthma. The major aim of this study was to assess the effects and the safety of nebulized GSH in patients with mild asthma.

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METHODS

Patients

We studied eight patients, five males and three females (age, 29 ± 7 [SD] yr), all nonsmokers who had mild asthma (FEV_1 , $88 \pm 11\%$ predicted FEV_1/FVC , $77 \pm 5\%$ predicted [13]). The diagnosis of asthma was based on a history of recurrent episodes of wheezing, chest tightness, and reversible airway obstruction previously documented by a physician. No attempt was made to differentiate between atopic and nonatopic patients. Their asthma was clinically mild with PD_{20} (provocative dose of methacholine producing a 20% fall in FEV_1) values ranging from 0.07 to 0.78 μmol ($0.32 \pm 0.31 \mu\text{mol}$), compared to normal values $> 1.9 \mu\text{mol}$. All patients controlled asthma symptoms by inhaling short-acting β -adrenergic agonists given as needed. In addition, two patients were taking budesonide; one, theophylline; and another, long-acting β -adrenergic agonist. These medications remained unchanged throughout the period of study. Short-acting β -adrenergics were withdrawn for 6 h before the study, while the rest of the medication was withheld for 12 h. No subject had a chronic illness other than asthma and none had had a chest infection or a major exposure to allergen within the previous 6 wk. All subjects volunteered for the study and gave informed consent in writing to participate. The protocol was approved by the Ethical Committee of Hospital Clínic, Universitat de Barcelona.

Glutathione Preparation

Glutathione solution ($600 \text{ mg} \cdot \text{vial}^{-1}$) was kindly provided by R. G. Crystal, M.D. (New York, NY), and reconstituted with 4 ml of 0.9% NaCl. We used this dosage based on previous studies (6) that showed an increased antioxidant effect on the airway fluid lining. Four milliliters of this solution were placed in the reservoir of a pneumatic nebulizer (Ultravent; Mallinckrodt, St. Louis, MO) that generates aerosol droplets appropriate for alveolar deposition. The size of aerosol droplets, determined by laser particle-size analysis, indicated a mass median aerodynamic diameter of 2.8 μm with a geometric SD of 1.3 μm . The nebulizer was driven at 40 psi (1 psi = 6.9 kPa) with compressed air to generate 10 $\text{L} \cdot \text{min}^{-1}$ of aerosol. Using a one-way valve, noseclip and mouthpiece in series, the system was closed; that is, all gas, including aerosolized GSH, was either inspired or expired through a filter to collect all expired drug. Using this system, aerosolization of 600 mg GSH to spontaneously breathing individuals with the nostrils occluded required 25 min. The pH and osmolarity of the GSH solution were 3.0 and 660 $\text{mosm} \cdot \text{kg}^{-1}$, respectively. Osmolarity was measured with an osmometer (Advanced Micro-Osmometer model 3MO-Plus; Advanced Instruments, Inc., Norwood, MA). Using this delivery system, Holroyd and co-workers reported (7) that the percentage of reduced GSH in the preparation was above 97% and remained unchanged following aerosolization.

Design

All studies were designed in a randomized, double-blind, cross-over placebo-controlled manner. Airway response was measured by assessing the resistance of the respiratory system (Rrs) using the forced oscillation technique applied at the mouth at a frequency range of 6–10 Hz (14) and by forced spirometry (Datospir 92; Sibelmed, Barcelona, Spain). Three sequential studies were designed (a) to examine the effects of GSH on airway caliber, (b) to evaluate the influence of osmolarity and pH of GSH solution, and (c) to analyze the role of pretreatment with salbutamol in influencing GSH challenge. The subjects inhaled the solutions at approximately the same time of the day, 1 wk apart, to avoid circadian interference. Bronchodilators were administered, if required, after the challenge. Each study lasted approximately 3 mo and the time elapsed between each study was at least 2 mo.

In the first challenge, patients inhaled a GSH solution or placebo (0.9% NaCl; osmolarity, 309 $\text{mosm} \cdot \text{kg}^{-1}$; pH, 6.0) to evaluate the effects of GSH on airway tone. Measurements of Rrs were done repetitively over a period of 20 min before and 25 min after completion of the challenge. Similarly, FEV_1 was measured before and 5 min after completion of GSH or placebo aerosolization. The final prechallenge Rrs value was the average of five individual measurements done at 4-min intervals. Because no differences were found in Rrs value after

challenge throughout the period of study, the final value corresponded to the average of seven measurements done at approximately 4-min intervals. An identical procedure was used for the two other studies.

In the second challenge, a similar protocol was developed to evaluate the effects of osmolarity and pH of GSH solution. In this case, placebo solution was made by adding HCl to 2% NaCl to obtain a pH of 3.0. This solution had similar osmolarity ($648 \text{ mosm} \cdot \text{kg}^{-1}$) and identical pH (3.0) to that of the GSH solution.

In the third challenge, patients received salbutamol (5.0 mg) or placebo (0.9% NaCl) from a pneumatic nebulizer (Oximask; Proclinics, La Llagosta, Spain) over 20 min before the GSH challenge. After salbutamol or placebo administration, FEV_1 and Rrs (two measurements) were assessed.

Finally, because of the possibility of sulfite generation during GSH nebulization due to the sulfhydryl group of cysteine, one of the aminoacids of GSH, we carried out an additional challenge, namely a dose-response study to inhaled metabisulfite. Metabisulfite challenge is one of the most widely used approaches to measure the airway's response to inhaled sulfites. We used the method described by Nichol and colleagues (15). Thus, the provocative dose of metabisulfite required to cause a 20% fall in FEV_1 (PD_{20}) was established.

Data Analysis

Results are expressed as mean \pm SD. Changes in FEV_1 and Rrs were analyzed using a two-way repeated measures analysis of variance (ANOVA). In the third challenge, *post hoc* comparisons were performed using paired *t* tests. The PD_{20} values of methacholine and metabisulfite challenges were log transformed for statistical correlations. Pearson's correlations were used when appropriate to assess relationships between variables. Statistical significance was set at $p < 0.05$.

RESULTS

Measurements performed at baseline of each challenge (means for 7 challenges: FEV_1 , $3.45 \pm 0.7 \text{ L}$, $92 \pm 10\%$ predicted; Rrs, $3.72 \pm 1.16 \text{ cm H}_2\text{O} \cdot \text{L}^{-1} \cdot \text{s}$) were within normal limits (FEV_1) (13) or slightly increased (Rrs) (14) and failed to reach significant differences among them (FEV_1 , $p = 0.07$; Rrs, $p = 0.41$).

Comparison between Glutathione and Placebo

All patients completed the study (Figure 1). However, in one patient (baseline FEV_1 , 2.68 L, 90% predicted; FEV_1/FVC , 75% predicted), GSH provoked marked bronchoconstriction. The FEV_1 decreased by -1.91 L (-69% from baseline), Rrs increased by $5.06 \text{ cm H}_2\text{O} \cdot \text{L}^{-1} \cdot \text{s}$ ($+154\%$ from baseline), and the patient had severe wheezing and breathlessness. The study was interrupted and nebulized salbutamol (5.0 mg) was immediately administered, producing rapid clinical improvement. This patient was excluded from the rest of studies. In the remaining seven patients, GSH induced cough in four and breathlessness in two. Inhaled salbutamol (400 μg) was given to these two patients at the end of the measurements with immediate full recovery. No symptoms were reported after the nebulization of placebo. The administration of placebo did not induce any variation in FEV_1 (change from baseline, $-0.02 \pm 0.18 \text{ L}$ [-1%]) and caused a marginal increase in Rrs (change from baseline, $+0.61 \pm 0.37 \text{ cm H}_2\text{O} \cdot \text{L}^{-1} \cdot \text{s}$ [$+17\%$]). By contrast with placebo, GSH induced marked bronchoconstriction, as shown by a decrease in FEV_1 of $-0.61 \pm 0.72 \text{ L}$ (-19% from baseline, $p = 0.03$) and an increase in Rrs of $2.16 \pm 1.62 \text{ cm H}_2\text{O} \cdot \text{L}^{-1} \cdot \text{s}$ ($+61\%$ from baseline, $p = 0.02$). For both measures, $n = 8$.

Comparison between Glutathione and Hypertonic Acid Solution

Glutathione provoked cough in four patients and breathlessness in two, who recovered completely with inhaled salbutamol; by contrast, the hypertonic acid solution (placebo) in-

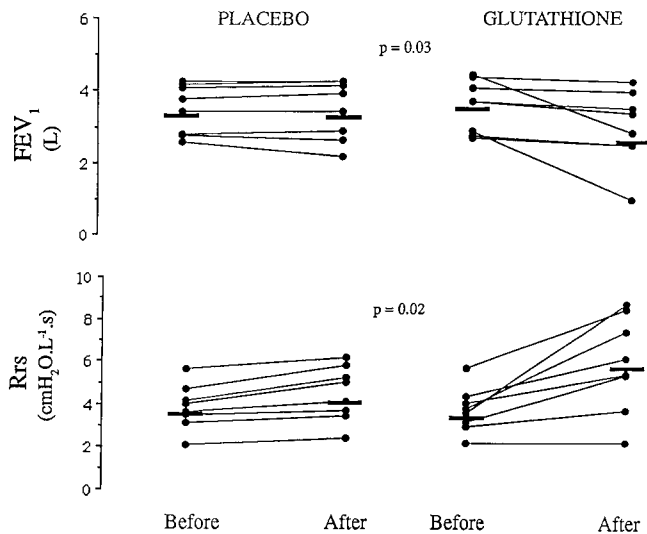


Figure 1. Individual responses of FEV₁ and Rrs to placebo or glutathione. Bars indicate mean values.

duced cough only in one patient. Compared with placebo, GSH challenge decreased FEV₁ by -0.40 ± 0.50 L (-11% from baseline, $p = 0.04$) and increased Rrs by $+1.95 \pm 1.25$ cm H₂O · L⁻¹ · s ($+51\%$ from baseline, $p = 0.02$), as shown in Figure 2. The nebulization of placebo altered both FEV₁ (change from baseline, $+0.13 \pm 0.09$ L [$+4\%$]) and Rrs (change from baseline, $+0.49 \pm 0.71$ cm H₂O · L⁻¹ · s [$+15\%$]), but less strikingly. Notably, GSH solution induced a similar degree of airway narrowing in this study and in the former one (Rrs, by $+51\%$ and $+48\%$, respectively; FEV₁, by -11% in both; $n = 7$, after excluding the patient who showed extreme bronchoconstriction in the first challenge).

Effect of Salbutamol on Glutathione Challenge

As expected, salbutamol inhalation produced mild bronchodilation (Figure 3). Compared with baseline, FEV₁ increased by $+0.29 \pm 0.23$ L ($+9\%$, $p = 0.02$), while Rrs decreased by -1.04 ± 0.79 cm H₂O · L⁻¹ · s (-25% , $p = 0.01$). By contrast, the nebulization of placebo produced no change in FEV₁

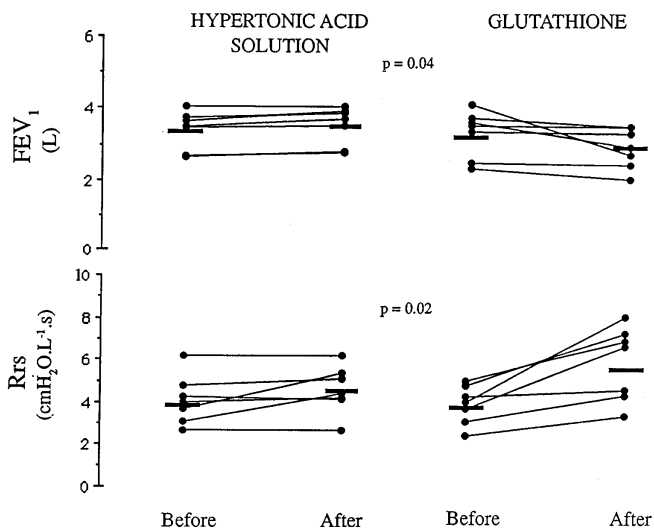


Figure 2. Individual responses of FEV₁ and Rrs to hypertonic acid solution or glutathione. Bars indicate mean values.

($+0.09 \pm 0.16$ L [$+3\%$]), whereas Rrs decreased moderately -0.53 ± 0.51 cm H₂O · L⁻¹ · s [-12% , $p = 0.03$]. The challenge with GSH after pretreatment with salbutamol still induced cough in two patients. However, compared with measurements performed after salbutamol, no significant change in FEV₁ ($p = 0.07$) nor in Rrs ($p = 0.21$) were shown after GSH challenge. By contrast, after pretreatment with saline placebo, GSH produced cough in three patients and mild breathlessness in one, inducing a significant increase in Rrs (change from placebo, $+0.89 \pm 0.78$ cm H₂O · L⁻¹ · s [$+24\%$], $p = 0.02$), yet FEV₁ remained essentially stable (change from placebo, -0.07 ± 0.19 L [-2%]).

Metabisulfite Challenge

We performed this study in six patients, who showed a dose-dependent decrease in FEV₁ with inhaled doubling concentrations of metabisulfite with minor side effects (cough in four). The mean PD₂₀ was 1.49 ± 1.83 μmol (range, 0.20–5.12 μmol), similar to other values shown previously (15, 16). A significant negative correlation was observed between airway hyperresponsiveness to metabisulfite and the GSH airway responses shown in the first two challenges (Figure 4) such that the greater the bronchoconstriction to inhaled GSH, the more intense the responsiveness to metabisulfite. By contrast, there was no correlation with methacholine bronchoprovocation.

DISCUSSION

The most novel finding of this study is that nebulized GSH at a dose of 600 mg had a bronchoconstrictor effect in this subset of patients with clinically stable mild asthma. This effect was repeatedly observed in the first three studies and was not related to osmolarity or pH of the GSH solution. Nebulized salbutamol prevented these deleterious functional effects.

It is notable that the GSH-induced airway changes in the third challenge were of lesser magnitude than in the other two, possibly because airway tone was conditioned by the previous nebulization with placebo, although changes in the degree of bronchial hyperresponsiveness by itself through the whole study also could play a role.

Glutathione has been administered safely at the same dose in patients with IPF and HIV-seropositive individuals, without adverse events. The former disorder is characterized by alveolar inflammation, increased release of oxidants, and decreased concentrations of the antioxidant GSH in respiratory ELF. Borok and colleagues (6) demonstrated that exogenous nebulized GSH in patients with IPF provoked an increase in total ELF GSH and oxidized GSH, with a decrease in spontaneous superoxide anion release by alveolar macrophages. Equally important, GSH is a metabolite or cofactor in several normal immune processes, so that a deficiency of reduced GSH can cause dysfunction of both lymphocytes and natural killer cells. As in patients with IPF, both systemic and ELF deficiency of GSH have been shown in HIV-seropositive patients. Because the lung is the most common site of infection in patients who progress to AIDS, it seems appropriate to suggest that increases of GSH levels in the ELF of these patients could improve local host defenses. Holroyd and colleagues (7) showed that nebulized GSH increased total GSH levels that remained within the normal range for at least 3 hr after treatment in HIV-seropositive patients. To our knowledge, however, GSH has never been administered to patients with bronchial asthma.

Several studies have evaluated the effects of acidity and/or high osmolarity of aerosol solutions in asthma and found that both cause cough and bronchoconstriction (17). Our results show that neither osmolarity nor pH account for the measured bronchoconstriction. Despite the fact that GSH solution is hypertonic

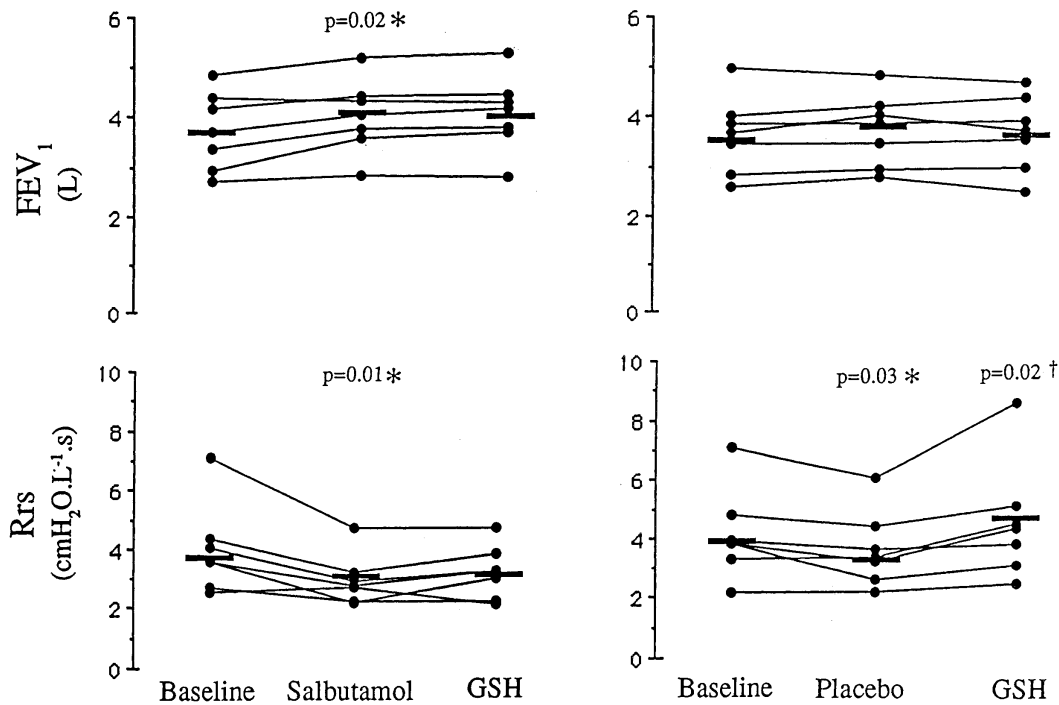


Figure 3. Individual responses of FEV₁ and Rrs to salbutamol or placebo and glutathione (GSH). Bars indicate mean values. *Comparing baseline with salbutamol or placebo. †Comparing GSH with placebo.

(660 mosm · kg⁻¹) and acidic (pH 3.0), the low volume of the solution given and the slow rate of administration (0.16 ml · min⁻¹) probably minimized the impact of these two factors. Anderson and Smith (17) recommended rates of aerosol delivery between 1.0 and 3.0 ml · min⁻¹ and solutions with osmolarity up to 875 mosm · kg⁻¹ for osmotic challenges in the assessment of bronchial hyperresponsiveness. The size of aerosol droplets generated by the pneumatic nebulizer used (2.8 μmol) was ap-

propriate for alveolar deposition, with only 25% of the solution expected to be deposited in the tracheobronchial tree.

To explain the bronchoconstrictor effect of nebulized GSH in our patients, we suggest two potential hypotheses. The most salient could be a result of the fact that GSH is a highly hydrophilic substance containing cysteine, an amino acid with a sulfhydryl group. When either sulfur species dissolves in aqueous solutions, a pH-dependent equilibrium is established among

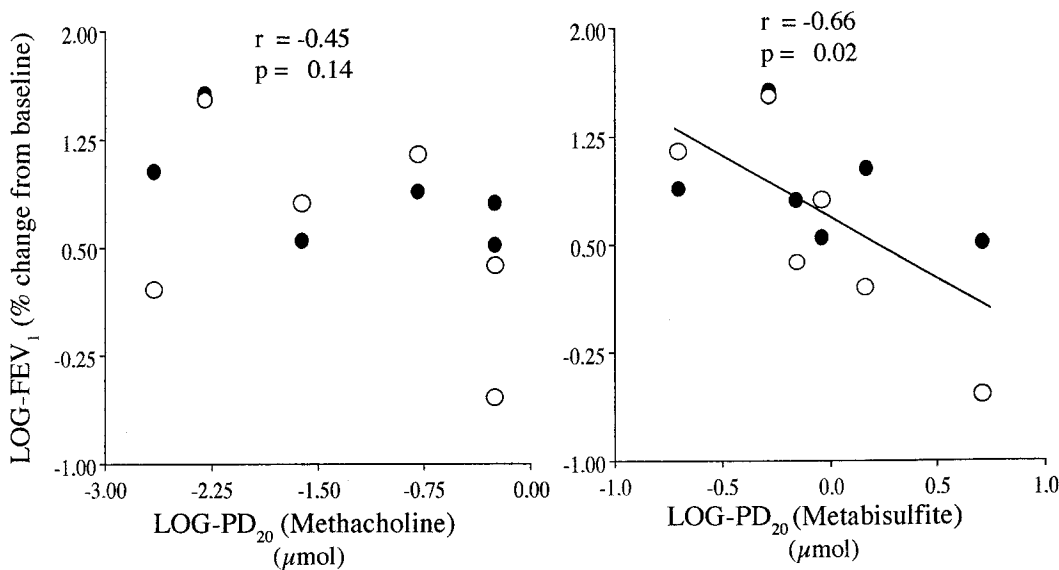


Figure 4. Individual plots of FEV₁ after GSH challenge (n = 6) against methacholine (left panel) and metabisulfite (right panel). Each patient is represented twice (closed circles = first challenge; open circles = second challenge). The close negative correlation shown between the responses to GSH and those to metabisulfite contrasts with the poor relationship shown after methacholine.

different sulfites (sulfur dioxide [SO₂], metabisulfite [SO₅⁼], bisulfite [SO₃⁻], sulfite [SO₃⁼]) (18). These sulfites produce the characteristic "rotten eggs" smell of GSH solution, and their acute bronchoconstrictor effects are well established. In healthy subjects, however, this effect was significant only after inhalation of concentrations in excess of 5 parts per million, or ppm (19). Several studies have shown that patients with asthma are exquisitely sensitive to the bronchomotor effects of sulfites (concentrations below 1 ppm). The precise mechanism of SO₂-induced bronchoconstriction remains elusive, and both cholinergic and noncholinergic mechanisms have been implicated (20, 21). Recently, a sensory nerve activation with tachykinins also has been invoked (22). It has been suggested that when sulfites are inhaled from a mouthpiece, as in our study, their bronchoconstrictor effects could increase (23). Although we did not measure the levels of sulfites during GSH nebulization, it is likely that they could be implicated in the induced bronchoconstriction. The finding that all the patients tested showed a significant bronchoconstrictive response to metabisulfite challenge, correlated inversely with the threshold of responsiveness to GSH, lends further support to the mechanism of bronchoconstriction induced by sulfite formation. The lack of adverse reaction to GSH nebulization in either IPF patients or HIV-seropositive individuals could be related to the different bronchoconstrictor sensitivity to sulfites of these populations.

A complementary explanation of GSH-induced bronchoconstriction may be that GSH is a versatile molecule that plays a key role in multiple metabolic pathways, one of them being the airway inflammatory response. Glutathione is involved in the metabolism, through conjugation, of leukotriene A₄, which results in the formation of leukotriene C₄ (LTC₄), which can be converted to leukotriene D₄ (LTD₄) (24, 25). Both LTC₄ and LTD₄ are well-known potent bronchoconstrictors with proinflammatory effects, such as increasing vascular permeability and microvascular leakage, and both have been invoked in the pathogenesis of bronchial asthma (26). The exogenous administration of GSH could provoke an imbalance in the components of ELF in asymptomatic asthmatic patients, thereby triggering an acute inflammatory reaction similar to that shown during an asthma attack.

The results of the third challenge could support, in part, these two contentions. On the one hand, β-adrenergic agonist agents can prevent sulfite-induced bronchoconstriction (19); on the other, we recently demonstrated an antiedema effect of salbutamol after platelet-activating factor (PAF) challenge in both healthy (27, 28) and asthmatic individuals (29), possibly by preferentially preventing airway microvascular leakage.

In conclusion, we have shown that nebulized GSH is deleterious to patients with stable mild asthma because it induces clinical symptoms, essentially cough, breathlessness, and marked functional bronchoconstriction that was prevented with nebulized salbutamol. Inhalation of sulfites that come from GSH solution could be involved in these effects. Our results should be considered if future therapeutic strategies with antioxidant preparations for patients with asthma or other chronic obstructive disorders are planned.

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