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## GLUTATHIONE PREVENTS THE EARLY ASTHMATIC REACTION AND AIRWAY HYPERRESPONSIVENESS IN GUINEA PIGS

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The prevalence of asthma has increased worldwide. The reasons for this rise remain unclear. Oxidative stress plays an important role in the pathogenesis of asthma. Glutathione (GSH) is the major representative of the class of nonprotein thiols and plays a pivotal role in a variety of enzymatic and nonenzymatic reactions that protect tissues against oxidative stress. In antioxidative reactions, GSH is converted into its oxidized form, glutathione disulfide (GSSG) that in its turn is enzymatically reduced into GSH to maintain a physiological redox balance. We used a guinea pig model of asthma to assess whether the early asthmatic reaction is associated with decreased lung levels of glutathione, and whether decreased glutathione is implicated in the increased airway smooth muscle reactivity that is associated with exposure of the lungs to allergen. Lung glutathione levels were decreased immediately after the onset of the early asthmatic reaction *in vivo* and associated with the release of 8-iso-PGF<sub>2α</sub>, an indicator for oxidative stress. Glutathione ethylester, a glutathione precursor, blunted the airway obstruction during an early asthmatic reaction in a perfusion model and glutathione depletion rendered the airways hyperreactive. Glutathione ethyl ester in the buffer prevented this hyperreactivity. These results indicate that glutathione can modulate the early asthmatic reaction as well as the airway hyperresponsiveness.

**Key words:** bronchoconstriction, glutathione, oxidants, asthma, prostaglandin, histamine, nitric oxide

### INTRODUCTION

There is growing evidence that oxidant stress plays an important role in the pathogenesis of asthma (1, 2) and COPD (3). It results in tissue damage, promotes inflammation and shifts redox balances towards oxidative reactions (4-6). In the past few decades, there has been significant increase in incidence and severity of allergic diseases. Environmental factors such as pollutants and food additives that do not have allergic potential but exert a pro-allergic adjuvant effect have been believed to play indisputable roles in the induction and/or exacerbation of allergic diseases (7). For this reason, identification of responsible factors and underlying mechanisms involved should be a research priority in order to make allergic diseases preventable.

The antioxidant, glutathione (GSH) is present in 15 mM concentrations in cultured human lung epithelial cells (8) which is considerably higher than the concentrations reported for most other cells (9). GSH concentrations in lung epithelial lining fluid *in vivo* are approximately 450 μM; this is 100-fold higher than in plasma (10).

As an antioxidant, GSH is converted into its homodimeric disulfide, oxidized glutathione (GSSG) (9). Increased formation of reactive oxygen species, resulting from inflammation, enhances formation of GSSG and decreases the tissue content of GSH (11).

Interestingly, the glutathione redox balance in lung tissues regulates hyperresponsiveness and airway inflammation in mice (12).

Although decreased GSH levels have been found in broncho-alveolar lavage (BAL) fluid in several lung diseases (13), the BAL fluid from stable asthmatics contains higher total glutathione levels than normal subjects (14). This increase is thought to reflect an adaptive response to the oxidant stress of inflammation (15).

It has been reported that GSH decreases bronchial smooth muscle contraction to different stimuli and that oxidant free radicals are produced during bronchial smooth muscle contraction (16). Moreover, GSH is known to influence rabbit and rat vascular (17, 18) and pig gastric (19) smooth muscle relaxation. We found in the guinea pig trachea that the hyperresponsiveness to histamine caused by removal of the epithelium is decreased by adding GSH to the organ bath (20).

To follow up on these findings, we tested the hypothesis that an early asthmatic reaction (EAR) is associated with decreased levels of GSH, and that the decreased GSH is implicated in the development of airway hyperresponsiveness. To test this hypothesis, it was investigated whether an EAR as provoked by allergen in sensitized guinea pigs decreased GSH levels in the lungs. Secondly, the effect on airway contractions of supplementing GSH during an EAR was determined. Thirdly,

airway reactivity to histamine was measured in lungs of guinea pigs in which glutathione had been depleted by pretreatment with a GSH synthesis inhibitor.

## MATERIALS AND METHODS

### *Ovalbumin sensitization*

Male specific pathogen-free guinea pigs (Dunkin Hartley, Harlan Nederland, Horst, The Netherlands) weighing 200–250 g body weight were sensitized by injecting a mixture of 20 µg OVA (grade V, Sigma, St Louis, MO) and 200 mg of the adjuvant, Al(OH)<sub>3</sub> (Merck, Darmstadt, Germany), in 1.0 ml saline. Al(OH)<sub>3</sub> in saline was used for the controls. Each animal received six injections: 0.5 ml was injected intraperitoneally, and five injections (0.1 ml each) were made subcutaneously in the axillar and inguinal regions and in the nuchal area. Three weeks after sensitization, airways were challenged with OVA according to different challenge protocols as described in the appropriate sections below.

### *Effluent analysis*

Buffer that exited the lungs during the development of contractile responses was collected. In the case of non-sensitized animals, buffer was collected at the corresponding time. Samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. Within three weeks after collection, levels of 8-iso-PGF<sub>2α</sub> were measured in the samples with commercial EIA kits (Cayman, Ann Arbor, MI for 8-iso-PGF<sub>2α</sub>) according to manufacturer's instructions.

### *Effect of an early asthmatic response on glutathione levels in the lungs*

OVA- and sham-sensitized animals were placed individually in a plexiglas box and exposed to a nebulized solution of OVA (0.01% in saline) until visual signs of an EAR (breathing with difficulty, legs lifting the body higher than normal, eyes turning darker) became apparent (typically after approximately one minute of OVA exposure). At that time point, animals were given an overdose of pentobarbital sodium (1 g/kg body weight, intraperitoneally) and were sacrificed within two minutes. Lungs were isolated immediately and snap frozen in liquid nitrogen until analysis for GSH/GSSG.

### *GSH depletion in vivo*

Male Hartley strain guinea pigs (200–220 g, Charles River Laboratories, Massachusetts, USA) were depleted of GSH using D,R-buthionine-L-sulfoximine (BSO, Sigma, St Louis), an inhibitor of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH biosynthesis (21). BSO was dissolved in PBS and 2.5 mmole/kg body weight was administered subcutaneously twice daily on four consecutive days. Controls received PBS only. As opposed to controls, BSO-treated animals did not gain weight in the course of treatment, but apart from that, appeared normal. On day 5, the lungs were isolated for perfusion studies.

### *Tracheally perfused lung preparation*

Tracheal perfusion was performed as previously described (20). Guinea pigs were anesthetized by intraperitoneal injection of 10% (w/v) urethane (2 ml/100 g body weight). When a sufficient level of anesthesia was achieved, a 2-cm-long polyethylene tube (1.67 mm I.D., 2.42 mm O.D.) was inserted into the trachea, the abdominal cavity opened, 500 U of heparin

injected into the vena cava, and 3 min later the abdominal aorta was severed. The thoracic cavity was opened to remove heart and lungs en bloc. The lungs were dissected free and hung in a plexiglas box at 37°C, 100% relative humidity. The lungs were perfused *via* the tracheal cannula with a phosphate-buffered physiological solution (pH 7.4) of the following composition: NaCl 137 mM, CaCl<sub>2</sub> 1.8 mM, MgCl<sub>2</sub> 1.05 mM, KCl 2.68 mM, NaHCO<sub>3</sub> 0.6 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.13 mM, and Na<sub>2</sub>HPO<sub>4</sub> 0.896 mM. The buffer was warmed to 45°C and pumped at a rate of 2 ml/min through a bubble trap before being cooled to 37°C for the actual lung perfusion. When the lungs had fully expanded, the buffer was allowed to exit the lungs through multiple small holes made in the pleura. The “back pressure” resulting from the perfusion (airway opening pressure, Pao) was recorded from a side tap at the tracheal cannula with the use of a pressure transducer. We have previously shown that during continuous flow, the Pao reflects the contractile state of the lung (20). Subsequently, lungs were perfused for 10 min to give a stable baseline pressure. At that time, different compounds were added to the buffer to test their effect on airway resistance as described hereafter.

For the induction of an EAR, 3.0 mg OVA in 0.30 ml perfusion buffer was injected in the perfusion system. Lungs contracted almost instantaneously upon the allergen injection as shown in a representative tracing (*Fig. 1A* and *B*). To control for allergen specificity of the EAR, a few lungs were challenged with bovine serum albumin followed by OVA.

As a measure of the airway response, the area under the Pao curve from the OVA infusion to the time of maximum resistance was recorded; values are expressed in kPa x s.

To study contractility of GSH-depleted lungs, increasing doses of histamine (10<sup>-9</sup>-10<sup>-5</sup> mole per dose) were added to the buffer. Because it was considered less important to express these responses as a function of time, they were expressed in kPa.

In some of the experiments described above, the cell-permeable GSH analogue GSH-ethyl ester (GSEt; Sigma, St Louis, MO; 1.0 mM) was dissolved in the perfusing buffer to increase or restore GSH levels.

At the end of each experiment, lungs were snap-frozen for GSH/GSSG analysis. In experiments with GSEt-perfused lungs, the lungs were flushed with 20 ml buffer without GSEt to remove any GSEt that had not been incorporated by the lung tissue. All experiments were approved by the appropriate ethical committees.

### *Measurement of GSH and GSSG in the lungs*

The snap-frozen superior lobe of the left lung was crushed in liquid nitrogen using a mortar and pestle; the resulting powder was divided between two eppendorf tubes. In one tube, a mixture of 1 M HClO<sub>4</sub> with 2 mM EDTA was added (1 ml per 250 mg powder) to assay total glutathione (GSH+GSSG), while the same mixture supplemented with 10 mM N-ethyl maleimide was added to the other tube to assess levels of oxidized glutathione. After vigorous vortex mixing, tubes were centrifuged at 5000 g for 10 min and total and oxidized glutathione concentrations in the supernatants were determined with a modified version (23) of the glutathione reductase-DTNB recycling assay according to Akerboom *et al.* (24). Values were expressed in nmole per g tissue (wet weight).

### *Statistics*

Data are expressed as mean±SEM. For all experiments, significance calculations were performed using the two-tailed Student's t-test. In the case of dose-response curves, maximum responses (E<sub>max</sub>) were tested for significance. Differences were considered statistically significant if P<0.05.

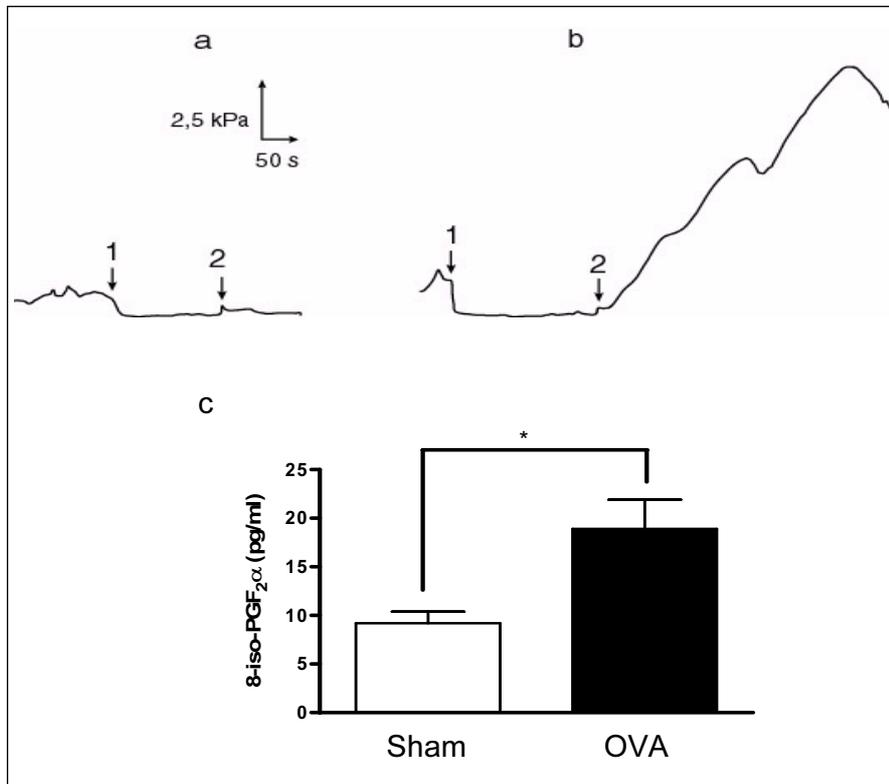


Fig. 1. Representative tracings of the airway opening pressure (Pao) during an allergen-induced early asthmatic reaction (EAR). Lungs from sham- (A) or OVA- (B) sensitized guinea pigs were challenged *ex vivo* with OVA in the perfusion buffer. The EAR-induced contraction of the lungs increased the Pao, while OVA challenge of a control lung hardly evoked any response. 1. Decrease in Pao caused by punching holes in the pleura; 2. The addition of OVA in the perfusion system results in a small temporary increase in Pao, which, in OVA-sensitized animals, is followed by an airway contraction. (C) Perfusate samples taken during the allergen-induced early asthmatic response of isolated guinea pig lungs and 8-iso-PGF<sub>2α</sub> levels were determined in the supernatants with a commercial EIA kits. White bar represent saline treated controls (sham), Black bar represent OVA sensitized and challenged animals. Data are means±SEM (n=6). \* represents a significant difference compared to sham treated animals (\*p<0.05).

## RESULTS

### *The early asthmatic reaction and the production of 8-iso-PGF<sub>2α</sub>*

For the induction of an EAR, 3.0 mg OVA in 0.30 ml perfusion buffer was injected in the perfusion system. Lungs contracted almost instantaneously upon the allergen injection as shown in a representative tracing (Fig. 1A and B). To control for allergen specificity of the EAR, a few lungs were challenged with bovine serum albumin which did not give any reaction. As an indicator for oxidative stress 8-iso-PGF<sub>2α</sub> was measured in the effluent during the EAR. The concentrations of 8-iso-PGF<sub>2α</sub> were more than doubled in the OVA sensitized and challenged group compared to the control group (Fig. 1C).

### *Effect of the early asthmatic reaction on lung GSH and GSSG levels*

Immediately after the onset of an EAR as induced by respiratory OVA challenge *in vivo*, lungs contained 40% less GSH (p<0.005) and three-fold more GSSG (p<0.005) than lungs from identically challenged sham-sensitized guinea pigs (Fig. 2). Consequently, the average amount of lung GSSG relative to total glutathione increased markedly from 5.5% in controls to 22.7% during the EAR (p<0.0001).

### *Airway resistance during the EAR in perfused lungs*

Challenge of lungs from sham-sensitized animals with OVA in the perfusion set-up evoked no significant increase in perfusion back pressure (Fig. 3), while OVA-challenge of the lungs of sensitized animals markedly increased perfusion back pressure (p<0.01). When GSEt (1.0 mM) was present in the buffer, the increase in resistance of lungs from OVA-sensitized animals was substantially (p<0.01) reduced.

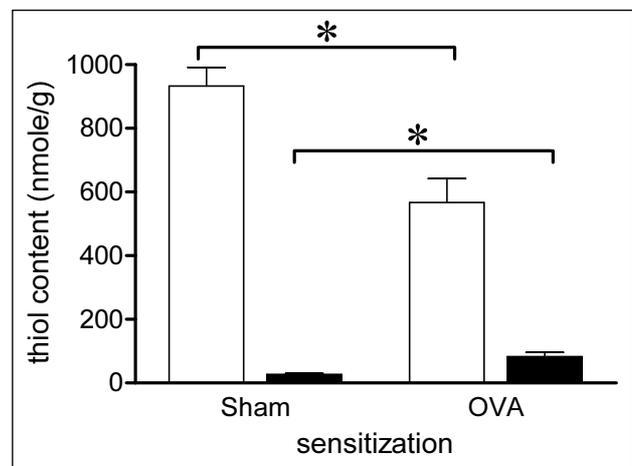


Fig. 2. Effect of an EAR on lung glutathione levels. Sham- or OVA-sensitized guinea pigs were challenged with an OVA aerosol, after which lungs were harvested and after snap-frozen the superior lobe of the left lung was crushed in liquid nitrogen; the resulting powder was divided between two eppendorf tubes and amounts of GSH and GSSG were measured. White bars represent the amounts of GSH and black bars are represents the amounts GSSG from saline treated controls (sham) and OVA-sensitized animals. Data are means±SEM (n=8). \* represents a significant difference compared to sham treated animals (\*p<0.05).

Notably, GSEt virtually nullified the difference between the OVA-induced airway responses of the sensitized and the sham-sensitized groups (p=0.06). Lungs of OVA-sensitized animals showed no response to challenge with BSA, while a subsequent challenge with OVA resulted in typical response (data not shown).

*Effect of in vivo GSH depletion with BSO on lung GSH and GSSG levels*

BSO treatment of guinea pigs decreased lung GSH levels by 85% (Fig. 4A,  $p < 0.005$ ), while GSSG levels were no longer detectable. GSEt-perfusion restored lung GSH and GSSG values in BSO-treated lungs to 48 and 50%, respectively, of the levels in non BSO-treated animals (Fig. 4B). Since in the GSEt treated group the differences in GSH and GSSG levels between BSO-

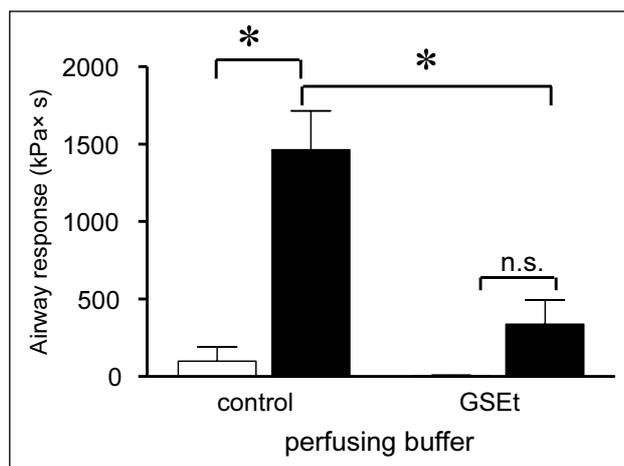


Fig. 3. Effect of an EAR on airway opening pressure (Pao) in the absence or presence of the GSH donor (GSEt), in the perfusing buffer. The lungs were perfused *via* the tracheal cannula with a warm physiological salt solution and pumped through a bubble trap for the actual lung perfusion. When the lungs had fully expanded, the buffer was allowed to exit the lungs through multiple small holes made in the pleura. The “back pressure” resulting from the perfusion was recorded. Cell-permeable GSH analogue GSH-ethyl ester GSEt was dissolved in the perfusing buffer to increase or restore GSH levels. The airway responsiveness was measured as described in material and methods. White bars are representative for saline treated (sham) animals and black bars are representative for OVA-sensitized animals. Data are expressed as means $\pm$ SEM ( $n=7$ ). \* represents a significant difference compared to sham treated animals (\* $p < 0.05$ ).

treated and control animals were not significant ( $p = 0.087$ ), GSEt perfusion actually eliminated the effects of BSO treatment.

*Effect of GSH depletion in vivo on histamine reactivity of isolated lungs*

Histamine induced dose-dependent contractions of control lungs, reaching a maximum Pao of  $9.2 \pm 1.4$  kPa following  $10^{-5}$  mole of histamine. This value was not changed by concomitant GSEt perfusion. Compared to the controls, GSH-depleted lungs from BSO-treated animals were hyperresponsive to histamine (Fig. 5A), reaching a maximum Pao of  $13.0 \pm 1.3$  kPa ( $p < 0.05$ ). GSEt in the perfusing buffer prevented BSO-induced increases in histamine responsiveness (Fig. 5B).

## DISCUSSION

This study shows that GSH is markedly depleted and GSSG is significantly increased during an EAR. In view of this shifted redox balance, an EAR is very likely to be accompanied by massive oxidative stress. Our data are the first report that GSH levels are acutely decreased during an experimental EAR. The acute GSH depletion and bronchoconstriction that accompany the allergic reaction are probably causally related, since perfusion with the GSH donor, GSEt, prevented allergen-induced airway smooth muscle contraction. In addition, lungs depleted from GSH by BSO treatment were hyperresponsive to histamine, an effect that could be reversed by addition of GSEt.

The occurrence of oxidative stress during the primary EAR is interesting, since inflammatory cells have not yet been recruited to the lungs as a result of prior respiratory allergen challenge. Although the source of the oxidative burst is unknown, histamine release by allergen-activated mast cells, a major feature of an EAR, is known to be accompanied by acute oxidative stress associated with enzymatic and non-enzymatic peroxidation reactions *in vitro* (25). Moreover, histamine release from cultured rat mast cells appeared to require oxidation of arachidonic acid (AA) by oxygen-centered free radicals (26). Allergen-induced mast activation could thus result in a self-amplifying cascade in which oxidative stress and the release of the smooth muscle contractant histamine are key events.

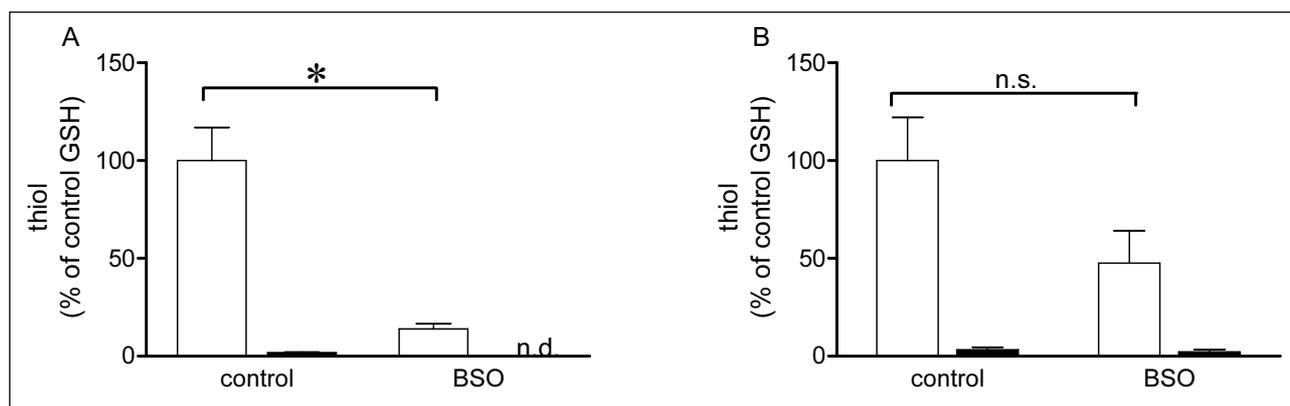


Fig. 4. Effect of *in vivo* GSH depletion with BSO on lung GSH and GSSG levels. Male guinea pigs were depleted of GSH using by using BSO, an inhibitor of  $\gamma$ -glutamylcysteine synthetase. BSO was dissolved in PBS and 2.5 mmole/kg body weight was administered subcutaneously twice daily on four consecutive days. Controls received PBS only. On day 5, the lungs were isolated for perfusion studies. A) BSO treatment decreased guinea pig lung GSH levels (white bars;  $n = 10$ ) by 85% compared to control animals ( $n = 8$ ), while GSSG (filled bars) in BSO-treated animals could no longer be detected. \* represents a significant difference compared to controls (\* $p < 0.05$ ). B) Perfusion with GSEt partially restored lung GSH and GSSG levels. Data are expressed as means $\pm$ SEM ( $n = 7$ ). n.d., not detectable; n.s., not significantly different.

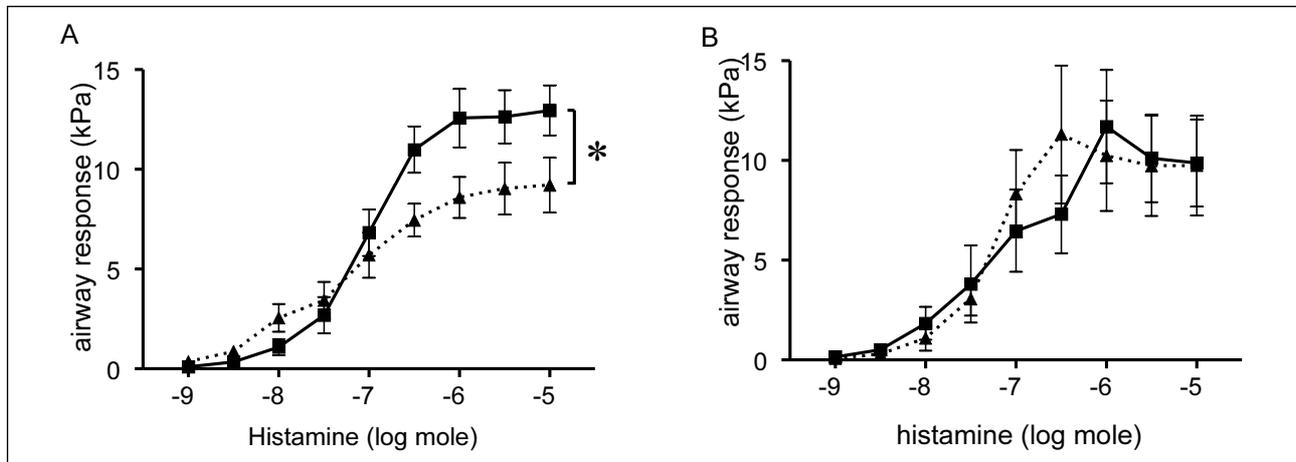


Fig. 5. Effect of GSH depletion on histamine reactivity of isolated lungs. Histamine induced dose-dependent contractions of control lungs, reaching a maximum Pao of  $9.2 \pm 1.4$  kPa following  $10^{-5}$  mole of histamine. This value was not changed by concomitant GSEt perfusion. Compared to the controls, GSH-depleted lungs from BSO-treated animals were hyperresponsive to histamine (A), reaching a maximum Pao of  $13.0 \pm 1.3$  kPa ( $p < 0.05$ ). GSEt in the perfusing buffer prevented BSO-induced increases in histamine responsiveness (B).

Interestingly, it was shown that the formation of histamine-releasing substances from AA by oxygen-centered free radicals can be inhibited by several free radical scavengers, including GSH (26). This is in line with our findings that a cell permeable GSH-precursor inhibited airway contraction during the EAR. The identity of the oxidative AA products is obscure. A candidate that has gained increasing attention in the field of oxidative stress markers is 8-epi-prostaglandin  $F_{2\alpha}$  (8-epi-PGF $_{2\alpha}$ ), a mainly non-cyclooxygenase-derived prostaglandin that is abundantly produced during oxidative stresses (27). 8-Epi-PGF $_{2\alpha}$  is an airway smooth muscle contractant (28, 29) and was found to be elevated in plasma (29) and exhaled breath condensate (30) of asthmatics. Indeed we did find the production of this mediator during the EAR *in vitro*, indicating that oxidative stress occurs during the OVA-induced airway constriction. Moreover, airway responses to the contractile agent that was studied in the current paper, histamine, was shown to be increased by GSH depletion. Since histamine is released from allergen-activated mast cells, the increased responsiveness to histamine under conditions of low GSH suggests an additional way to explain increased airway contraction during an EAR.

Apart from affecting the production or action of smooth muscle contractile agents, GSH may influence relaxant substances. GSH is known to enhance the bioavailability of nitric oxide (NO) (31), a major airway smooth muscle relaxant that, moreover, antagonizes histamine release by mast cells (32). Thus, depletion of GSH may increase airway contractility by hampering the effects of NO (16). Another airway smooth muscle relaxant is PGE $_2$ . PGE $_2$  synthesis was found to be downregulated in renal homogenates by limiting GSH concentrations (33). Unfortunately, an interference of GSH with the relaxant compounds NO and PGE $_2$  could not be substantiated because they were undetectable in the lung perfusate samples (experiments and data not shown).

An earlier report that total glutathione levels in lung lavage fluid are higher in stable asthmatics than in healthy subjects (14) seemingly contrasts with the present findings. However, acute depletion of GSH generally stimulates GSH production and results in enhanced GSH levels at a later stage in various cells and tissues (20), including cultured human lung epithelial cells (34). Thus, the enhanced GSH levels in stable asthmatics and COPD patients may be the result of GSH depletion by the

local inflammatory cells and possibly by preceding exacerbations (35, 36).

It has to be stressed however, that GSH is not the only antioxidant that is present in the respiratory tract. We cannot exclude that for instance vitamin C or super oxide dismutase participate as well (13).

In conclusion, the airway GSH levels in an animal model of an EAR are acutely decreased and associated with oxidative stress as indicated by the release of 8-iso-PGF $_{2\alpha}$ . Furthermore, decreased GSH is associated with enhanced airway responses. This is probably due to interference of GSH with different mechanisms, but at least due to interference at the level of airway smooth muscle reactivity to histamine. We speculate that maintenance of a physiologic redox balance in the airways during asthma could be an interesting novel therapeutic approach in the future.

*Abbreviations.* BAL: broncho-alveolar lavage; BSA: bovine serum albumin; BSO: D,R-buthionine-L-sulfoximine; EAR: early asthmatic reaction;  $E_{max}$ : maximum response; FEV: forced expiratory volume; 8-epi-PGF $_{2\alpha}$ : 8-epi-prostaglandin  $F_{2\alpha}$ ; GSEt: glutathione ethyl-ester; GSH: glutathione; GSSG: oxidized glutathione; NO: nitric oxide.

Conflict of interests: None declared.

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