Glutathione and airway function

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Function of the antioxidant effect of glutathione in the lungs, symbolized by the antioxidant action of a zinc anode on a ship's hull.

Glutathione and airway function

Glutathion en luchtwegfunctie

(met een samenvatting in het Nederlands)

Proefschrift

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General introduction

Allergic asthma

Allergic asthma affects at least 5% of adults and 10 - 15% of children in westernized countries (1). Characteristics of the disease include reversible airflow obstruction and increased airway responsiveness to a variety of stimuli. They are associated with airway inflammation in which eosinophils, mast cells, and lymphocytes, together with a multitude of cytokines and other signaling molecules, are important players (2). Allergen exposure of people at risk of developing asthma results in an inflammatory cascade that is initiated by dendritic cells. They are abundantly present in the submucosa of asthmatic airways and are essential for the uptake of allergens (3). After taking up allergen, dendritic cells migrate to local lymph nodes where they present the allergen to T-cells that subsequently differentiate into Th2 cells. These cells then secrete the cytokines that induce proliferation of, and IgE production by allergen-recognizing B cells. Antigen-specific IgE is bound by receptors on the cell surface of mast cells. Subsequent cross-linking of mast cell-bound IgE upon re-exposure to allergen induces release of mediators by the cells (see (4) for a review). Such mediators include histamine, prostaglandin D_2, and leukotrienes C_4, D_4, and E_4 (5). In general, mast cell-derived mediators produce airway constriction, increase microvascular permeability, enhance airway smooth muscle responsiveness and promote recruitment of eosinophils. In addition to secreting rapidly acting mediators, activated mast cells, together with T cells, release a variety of cytokines, promoting the chronic features of inflammation after allergen exposure, as reviewed in (6). These cytokines induce recruitment of eosinophils and lymphocytes to the airways and promote their survival and activation. In addition, a number of chemokines that attract and activate inflammatory cells are produced by structural airway cells such as epithelial and endothelial cells and fibroblasts (7), as well as smooth muscle cells (8). Thus, a variety of events are involved in triggering and maintaining an allergic inflammatory response in the airways, leading to the chronic inflammatory airway disease, clinically defined as allergic asthma.

Asthma and reactive oxygen species

A variety of cells in the lungs of asthmatics is capable of producing large amounts of reactive oxygen species (ROS). Activated eosinophils, neutrophils, monocytes, and macrophages can produce superoxide ($O_2^{\cdot -}$) (9). Subsequent dismutation of $O_2^{\cdot -}$ yields hydrogen peroxide (H_2O_2). Indeed, H_2O_2 levels in exhaled air are higher in asthmatics than in normals, and can be decreased by corticosteroid treatment (10).

Although both $O_2^{\cdot -}$ and H_2O_2 are only moderate oxidants, they can become potentially deleterious through their reactions with other molecules. For instance, hydroxyl radical ($OH^{\cdot}$) is a very powerful oxidant and requires $O_2^{\cdot -}$ for its formation. H_2O_2 is used as a substrate by myeloperoxidase (MPO) in neutrophils and monocytes (11), or eosinophil peroxidase (EPO) in eosinophils (12) to produce the highly oxidative hypohalous acids (HOCl and HOBr).

ROS, which may be radicals or non-radicals, serve in cell signaling as messenger molecules (13) and in host defense, as killers of pathogens or tumor cells (14). However, in case of a poorly functioning antioxidant system or if ROS are produced in excessive amounts, they may become harmful to host tissue, ultimately leading to tissue injury and inflammation (15). Indeed, the influx and activation of eosinophils into the lungs of asthmatics can induce substantial oxidative damage (16). Notably, eosinophils can generate more $O_2^{\cdot -}$ and H_2O_2 than neutrophils, and their EPO content is higher than the MPO content of neutrophils (17). In addition to recruited inflammatory cells, the structural airway cells such as epithelial cells can also be potent sources of ROS (18).

The inflammatory cells recruited to the asthmatic airways produce larger amounts of free radicals upon stimulation *in vitro* than do leukocytes obtained from healthy subjects (for a review, see (19)). The resulting oxidative stress may render airway smooth muscle hyperreactive to contractile stimuli (20), while long-term oxidative stress may play a role in airway remodeling in asthma (9). Exogenous sources of ROS may also induce airway hyperreactivity. For instance, ozone exposure renders guinea pig airways hyperreactive to methacholine (21) and increases the airway hyperreactivity of mild asthmatics (22). Hyperoxia induces airway hyperreactivity in neonatal guinea pigs, which is thought to mimic the hyperresponsiveness generated by hyperoxia during the treatment of respiratory distress syndrome (23).

Asthma and antioxidants

The lungs have a variety of both enzymatic and non-enzymatic antioxidant tools to combat oxidative stress. Numerous studies have addressed potential disturbances of anti-oxidant systems in asthma, but their outcomes are not always unambiguous. For instance, superoxide dismutase (SOD) activity was found to be similar in broncho-alveolar lavage (BAL) fluid of normals and asthmatics (24), higher in asthmatics than in normals (25), and lower in asthmatic BAL cells and bronchial epithelial cells as compared to those of normals (26). The findings pertaining to other antioxidant systems are less conflicting. Several publications have demonstrated that asthmatics have lower levels of selenium, required for glutathione peroxidase function (27-29).

Insufficient dietary intake of antioxidant vitamins by asthmatics has been reported (30-33), as well as low levels of vitamins C and E in their lungs (34). The antioxidant, glutathione, and its levels in asthmatics are addressed in separate sections below.

Glutathione

Glutathione (GSH, L-γ-glutamyl-L-cysteinylglycine) is the most abundant non-protein thiol in mammalian cells and a major (airway) antioxidant (35). As a substrate for GSH S-transferase, it is involved in the detoxification of xenobiotic compounds. More relevant to this thesis is the role of GSH as a substrate of GSH peroxidase in the detoxification of ROS and free radicals. These are also detoxified by GSH in non-enzymatic reactions. When participating in antioxidant reactions, two molecules of GSH react to form the disulfide, oxidized glutathione (GSSG). GSSG is readily reduced by glutathione reductase under normal conditions, so that tissue concentrations of GSSG are normally less than 3 to 5% of the total (GSH + GSSG) glutathione concentrations in the body (36). However, in conditions of oxidative stress, this balance may be disturbed. Therefore, the relative amount of GSSG is often taken as an indicator of oxidative stress.

GSH metabolism

As outlined in figure 1, cellular GSH synthesis takes place in the cytosol in two steps. In the first, rate-limiting step, γ-glutamylcysteine is formed from γ-glutamic acid and cysteine by γ-glutamylcysteine synthetase. In the second step, GSH synthetase catalyses the reaction between γ-glutamylcysteine and glycine to form GSH. Maximum cytosolic concentrations of GSH are limited via feedback inhibition of the first reaction step by GSH itself.

The γ-glutamyl bond in GSH makes the molecule resistant to intracellular peptidases. However, this bond can be cleaved by γ-glutamyl transpeptidase, an enzyme located on the external cell membrane of cells in various tissues, including lung tissue (37, 38). As indicated by Berggren *et al.* (39), γ-glutamyl transpeptidase is essential for the use of extracellular GSH to maintain cytosolic levels of GSH in lung tissue. Their studies in isolated and perfused rat lung showed that lung tissue utilizes plasma GSH in a process of extracellular breakdown and cytosolic resynthesis, rather than by direct uptake. The importance of GSH in the lung was further demonstrated by Martensson, who calculated that about 200 nmol/min of

GSH is taken up from plasma during transit through murine lung. For comparison, this value has been estimate to be 50 nmol/min in mouse kidney (37), another organ with a high GSH consumption (40).

Depletion of cellular GSH as a research tool

To study the role of GSH and GSH synthesis, experimental induction of GSH deficiency is a valuable tool. Several ways have been described to deplete GSH (for a review, see (41)). One way is to expose tissues to oxidizing agents such as diamide or *t*-butylhydroperoxide. Other strategies are the use of compounds that react with the thiol group of GSH, such as diethyl maleate and phorone. Both strategies, however, have disadvantages due to their toxic side effects and lack of specificity. The most specific way to deplete GSH is the use of amino acid sulfoximines, which are inhibitors of the rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase (figure 1). Although several sulfoximines are known, buthionine sulfoximine is most frequently used because of its low toxicity compared to the other compounds of its class.

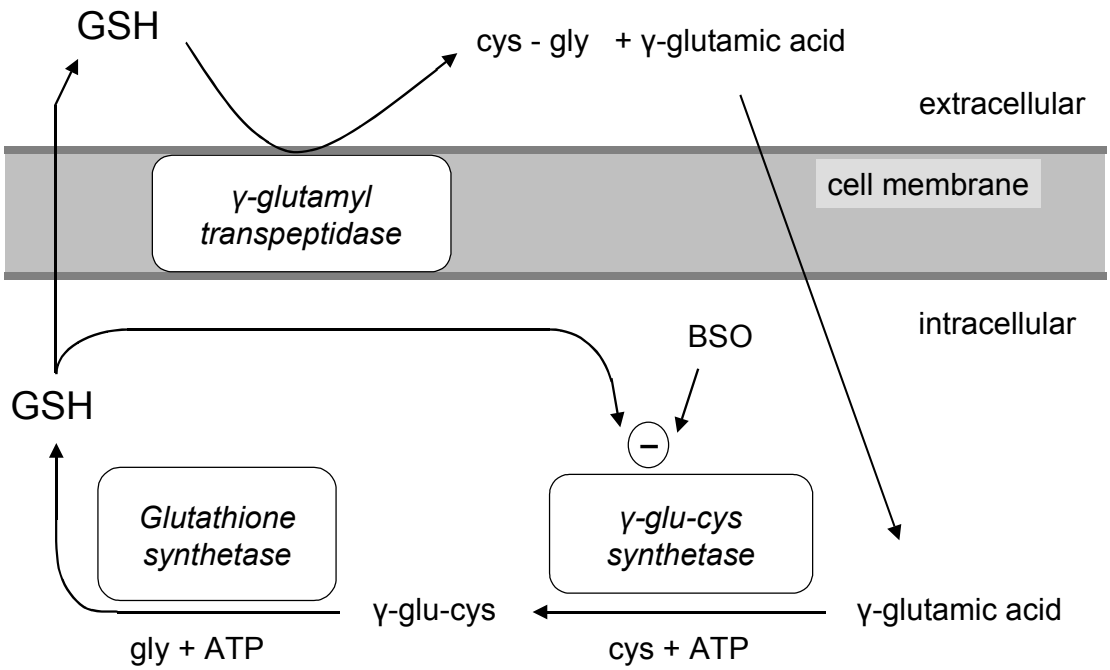

Figure 1. GSH synthesis. Modified from (48).

GSH and nitric oxide

The thiol (-SH) group of GSH accounts for most, if not all of the specific properties of the molecule. One of them is the capacity of GSH to bind the (airway) smooth muscle relaxant, (42) nitric oxide (NO), to form S-nitrosoglutathione (GSNO). Genuine NO has a short half life in biological systems, and GSNO is thought to act as a stable reservoir of NO. Although GSNO is often referred to as a NO-donor, the genuine compound has smooth muscle relaxing properties, similar to those of NO. The interactions of nitric oxide with thiol groups have recently been outlined in an excellent review by Gaston (43).

Glutathione in asthma

Physiologic GSH levels in alveolar lining fluid are about 400 μM, which is more than 100-fold higher than in blood plasma (44). Smith *et al.* have reported higher levels of glutathione in lining fluid of mild asthmatics than normals (24), but they made no distinction between GSH and GSSG. The increased levels of total glutathione may result from a rebound effect after an episode of oxidative stress. Indeed, when alveolar epithelial cells are exposed to oxidative stress *in vitro*, their GSH level initially drops, but later increases to levels higher than baseline (45). In contrast with the enhanced glutathione levels in lining fluid of asthmatic reported by Smith *et al.* (24), similar GSH and GSSG levels were found in induced sputum samples of asthmatics and healthy subjects (46). As far as known, no data are available on lung tissue levels of GSH and GSSG of asthmatics as compared to healthy individuals. For a review on GSH regulation in inflammatory lung diseases, including asthma, the reader is referred to (47).

Aim and outline of the present study

This thesis has addressed the relation between airway contractions and the physiology of one of the most prominent airway antioxidants, GSH, as prompted by accumulating evidence for an oxidative burden in asthmatic lungs, and by the fact that excessive airway smooth muscle contraction is a hallmark of asthma.
In chapter 2, it was investigated whether contractile differences between isolated proximal and distal trachea segments can be attributed to differential GSH content or to differential production or release of contractile and relaxant mediators.

The relaxing properties of GSH and other thiols in isolated and perfused guinea pig trachea have been demonstrated in chapter 3, and the question has been addressed which mediators of trachea tone are involved in GSH-induced tracheal relaxation.
Since the consequences of allergic inflammation and the associated oxidant burden in the airways for lung GSH levels are unclear, the effects of an early asthmatic response (EAR) *in vivo* on GSH concentrations in guinea pig lung tissue have been studied in chapter 4. Using a setup for perfusion of isolated lungs, the protective effect of exogenous GSH against excessive airway contractions during an EAR was shown. The effects of experimental GSH depletion in guinea pigs on contractile responsiveness to histamine of isolated and perfused lungs has been demonstrated in the same chapter.
Chapter 5 describes the effects of a tissue GSH enhancing diet on lung contractions during an EAR in a guinea pig model of allergic asthma. It was also investigated whether this diet affected allergen-induced concentrations of various contractile and relaxant mediators in the airways, as well as parameters of oxidative stress.
In chapter 6, a murine model of allergic asthma was used to address the possibility of an intervention by delivering GSH-donors to the lungs via aerosols. The aerosols consisted of GSH-ethyl ester or N-acetylcysteine and were delivered shortly before or during respiratory allergen challenges, or during methacholine challenges. The effect of these treatments on airway reactivity, as well as on lung GSH content, and recruitment of inflammatory cells into the airways has been examined.
Finally, in chapter 7, the results of the experimental studies as a whole are discussed in the context of related literature.

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2

Differential responsiveness of proximal and distal parts of isolated guinea pig trachea - glutathione is not involved

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Abstract

The present study addressed the question whether proximal and distal trachea segments of the guinea pig respond differently to contractile agents.
Using a perfused trachea set-up, application of histamine at the luminal side of the trachea induced similar contractions in proximal and distal trachea segments. However, a pharmacologically non-specific contractile stimulus, serosal KCl (50 mM), induced a 60% stronger contraction in proximal trachea as compared to distal trachea. Similarly, the increase of histamine-responsiveness as induced by removal of the epithelium (a mimic for epithelial sloughing in asthmatics) was significantly greater in proximal than in distal trachea segments (E_{max} values 3057 and 1526 mg, respectively). In epithelium-intact tracheas, inhibition of epithelial nitric oxide synthase increased histamine-induced contractions in proximal segments only, while inhibition of mucosal cyclo-oxygenase increased reactivity to histamine to the same degree in proximal and distal epithelium. In contrast, serosal cyclo-oxygenase inhibition increased histamine-reactivity in proximal trachea segments only (E_{max} 2690 and 5180 mg without and with cyclo-oxygenase inhibitor, respectively). Levels of glutathione, a compound that has moderating effects on airway contractions, were similar in proximal and distal trachea segments. The results indicate that the higher intrinsic contractility of proximal trachea segments is counteracted by epithelial nitric oxide synthase and serosal cyclo-oxygenase.

Introduction

One of the hallmarks of asthma is increased responsiveness of the airways to contractile mediators such as histamine (1). Airway contractions are widely studied in isolated parts of the airways. There is abundant evidence for contractile reactivity differences between upper and lower airways, but such differences within the tracheal tube are less well documented. However, *in vitro* experiments are often performed on multiple pieces of tissue from the same trachea. Hence, for practical reasons, it is important to know whether there are reactivity differences between different parts of the trachea. In addition, investigation of possible differences may shed new light on the relative role of factors defining tracheal responsiveness.
In our laboratory, an organ bath system with perfused isolated guinea pig tracheas is used. To reduce the use of experimental animals, we generally separate each trachea into a proximal and a distal part. Trachea segments without epithelium are frequently used as a model to mimic epithelial sloughing (2) as seen in asthmatics (3). Using this model with histamine as a contractile agent, we observed contractility differences between proximal and distal parts of guinea pig tracheas. This was further investigated by using tracheas with and without epithelium. Since the contractility differences were especially pronounced in the absence of the epithelium, the role of two histamine-induced, epithelium-derived relaxing factors, nitric oxide (NO) (4) and prostaglandin E_2 (PGE_2) (5), was studied. Further, we studied whether GSH is involved in the contractile differences, since GSH is abundant in airway epithelium (6) and capable of counteracting airway contractile responses (chapters 3, 4, 5, and 6).

Methods

Animals
Male specific pathogen-free Dunkin Hartley guinea pigs weighing 350 - 400 g (Harlan Nederland, Horst, The Netherlands) were housed under controlled conditions. Water and commercial chow were allowed *ad libitum*.

Isolation of tracheal segments
Guinea pigs were sacrificed with an overdose of pentobarbitone-sodium (Nembutal®, 0.6 g kg^{-1} body weight, *i.p.*). Tracheas were dissected free of connective tissue and blood vessels, isolated, and divided in a proximal and distal part as follows: the part of the trachea that contained the first 14±1 cartilage rings from the larynx was designated proximal, and the part containing the following 14±1

rings was designated distal parts of the trachea. Typically, 3 or 4 rings separated the latter part from the bifurcation, but were not included because of practical reasons relating to the isolation procedure. Two steel hooks were inserted through opposite sites of the tracheal wall with the smooth muscle between them as described before (2). The hooks comprised three cartilage rings; therefore, the sites of proximal and distal parts where force development was actually recorded were separated by 11±2 cartilage rings. Where indicated, the epithelial layer was removed from the tracheal segments by gently rubbing with a cotton swab as described earlier (2).

Perfused organ baths

Trachea segments were mounted in perfused organ baths according to a modified method of Pavlovic (7). The organ baths contained Krebs buffer of the following composition (mM): NaCl (118.1), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaHCO_3$ (25.0), K_2HPO_4 (1.2), glucose (8.3). The mucosal side of the trachea was perfused with Krebs solution independently from the serosal side by means of a peristaltic pump delivering a flow rate of 2 ml/min. The Krebs solution was continuously gassed with 5% CO_2 in O_2 and kept at 37°C. One hook was fixed to the bottom of the organ bath; the other hook was attached to an isometric force transducer (Harvard Bioscience, Kent, UK). Transducers were connected to an analogue-digital converter, delivering digital signals to a computerized set-up. The set-up allowed continuous sampling, on-line equilibrium detection, and real-time display of the responses on a computer screen.

The tracheal tension was set at an optimum counter weight of 2.0 g. The tissues were allowed to reach a stable tone for 60 min, during which the buffer was refreshed every 15 min. If necessary, tissues were allowed additional time to equilibrate without the buffer solution being changed.

Contractile stimulation; pharmacological modulation of contractions

Non-specific trachea smooth muscle responses were evoked by adding KCl at a final concentration of 50 mM to the serosal buffer. For pharmacologically specific contractile responses, tracheas were stimulated at the mucosal side with cumulatively increasing concentrations of histamine (10^{-8} to 10^{-3} M), or with a single concentration (10^{-3} M) of histamine at the serosal side of the tracheal tube.

NO synthase (NOS) was inhibited by L-NAME (150 μM, final concentration), which was administered after the equilibration period, but 20 min before starting the histamine series. Tracheas with epithelium received the NOS-inhibitor in the mucosal buffer only, whereas epithelium-denuded tracheas were treated both serosally and mucosally. Controls received the same concentration of D-NAME; the compounds remained in the buffer throughout the experiment. The cyclo-oxygenase

inhibitor, indomethacin (1.0 μM, final concentration), was administered in a similar fashion; controls received vehicle (Tris buffer, 2.5 mM, final concentration).

GSH/GSSG measurements

Tracheas were isolated from the animals, divided in a proximal and distal part as described above, and snap frozen in liquid nitrogen. Three segments per sample were crushed using a mortar and pestle; the resulting powder was divided between two eppendorf tubes. In one tube, a mixture of 1 M $HClO_4$ 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 (NEM) 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 (8) of the glutathione reductase-DTNB recycling assay according to Akerboom *et al*. (9). Values were expressed in nmole per g tissue (wet weight).

Statistics

If concentration response curves showed a clear plateau, E_{max} values were compared with the Student's two-tailed T-test. This test was also used to analyze the data on tracheal GSH and GSSG content. If no clear plateau was reached, the curves were analyzed with a repeated measures test. P-values < 0.05 were considered to reflect significant differences.

Results and discussion

Mucosal perfusion with histamine of tracheas with intact epithelium resulted in concentration-dependent increases of reactivity that were similar for proximal and distal segments at all concentrations, except at the highest (1 mM) (figure 1). At the latter concentration, stronger contractions were observed in proximal segments than in distal ones.

Moreover, when intact tracheal tubes were stimulated non-specifically at the serosal side with KCl (50 mM), proximal segments showed stronger contractions than distal segments as well (figure 2). Apparently, the intrinsic contractile capacity of tracheal smooth muscle is higher in proximal than in distal tissue. Combining the results of figures 1 and 2, we hypothesized that in intact tracheal tissue, the epithelium of the proximal trachea conceals the higher contractile potential of proximal segments. To test this hypothesis, contractility of tracheal tubes without epithelium to histamine in the mucosal buffer was assessed.

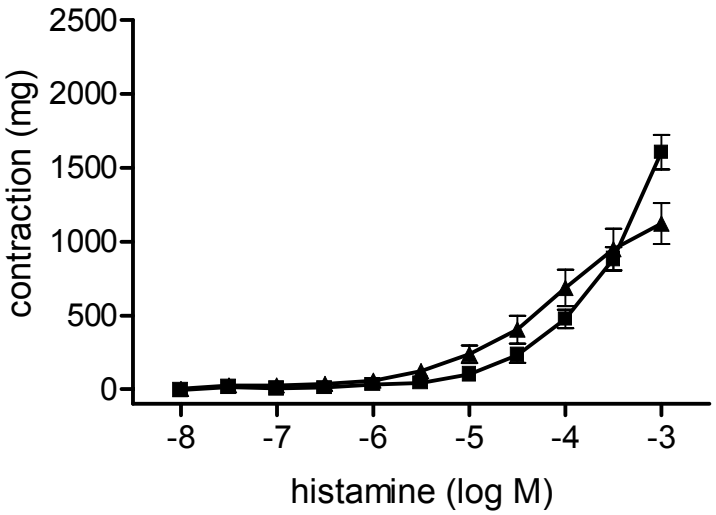

Figure 1. Contractions of proximal (squares) and distal (triangles) guinea pig trachea segments with intact epithelium following exposure to increasing histamine concentrations administered at the mucosal side. Proximal and distal segments respond differently to the highest concentration of histamine only. N = 9 trachea segments per group.

Results showed that epithelium-denuded proximal tubes exhibited substantially higher contractions than similar distal ones throughout the concentration range (figure 3; E_{max} 3057 vs. 1526 mg; $P < 0.0005$).

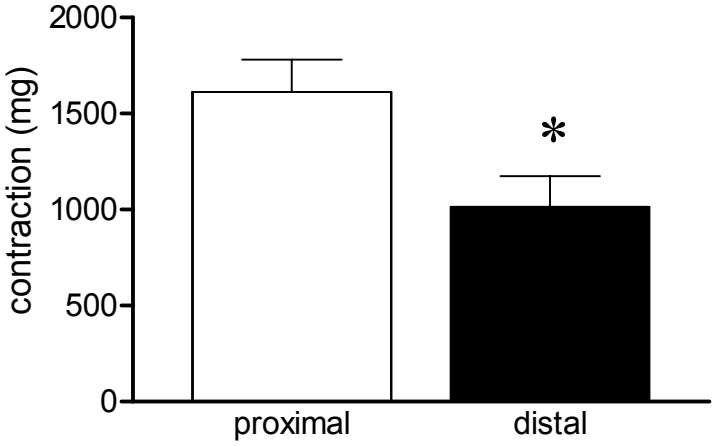

Figure 2. Contractions of proximal and distal guinea pig trachea segments with intact epithelium upon stimulation with 50 mM KCl at the serosal side. Proximal segments contract more than distal segments (*, $P < 0.05$). N = 9 trachea segments per group.

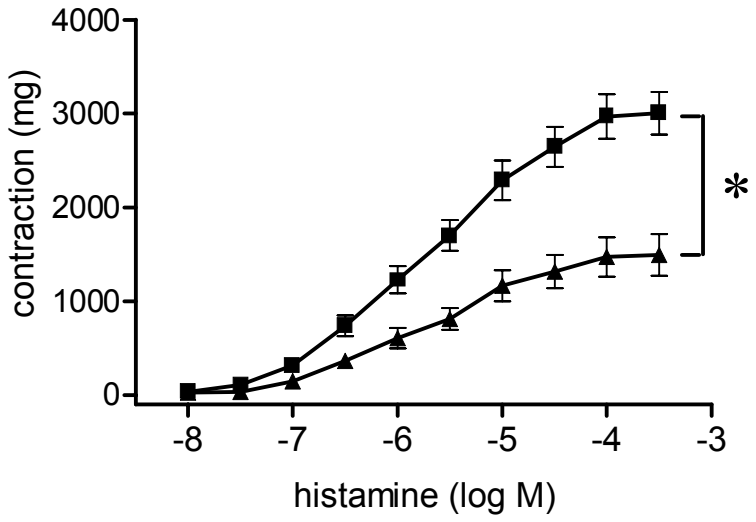

Figure 3. Contractions of proximal (squares) and distal (triangles) guinea pig trachea segments without epithelium following exposure to increasing histamine concentrations at the mucosal side. Proximal segments are more responsive to histamine than distal segments (*, $P < 0.0005$). N = 9 trachea segments per group.

The findings in figure 3 can not be entirely explained by the notion that the overall stronger contractility of epithelium-denuded tracheas as compared to tracheas with epithelium (figure 1) accentuates proximal-distal contractile differences. Notably, in intact tracheas, contractions of proximal and distal segments are similar for contractions up to 1000 mg (figure 1) whereas differences between proximal and distal segments in epithelium-denuded tracheas are obvious even at contractions far below 1000 mg (figure 3). Thus, assuming that epithelial permeability to histamine is the same in proximal and distal segments, we concluded that a relaxing factor in the epithelium of the proximal trachea compensates for the higher intrinsic smooth muscle contractility in proximal trachea segments.

In the light of these considerations, the role of one of the major epithelium-derived relaxing substances in the airways, NO (10, 11), was studied. It has been reported that tracheal NO is produced by the epithelium (12) and is implicated in the functional antagonism of histamine-induced airway contractions (4, 13, 14). If this would be the case mainly in the proximal trachea, inhibiting epithelial NOS should have a more pronounced effect in proximal than in distal trachea segments. The NOS inhibitor, L-NAME, had no effect in distal segments but tended to increase the response to histamine in the proximal segments throughout the concentration response curve as compared to the inactive enantiomer D-NAME (figure 4).

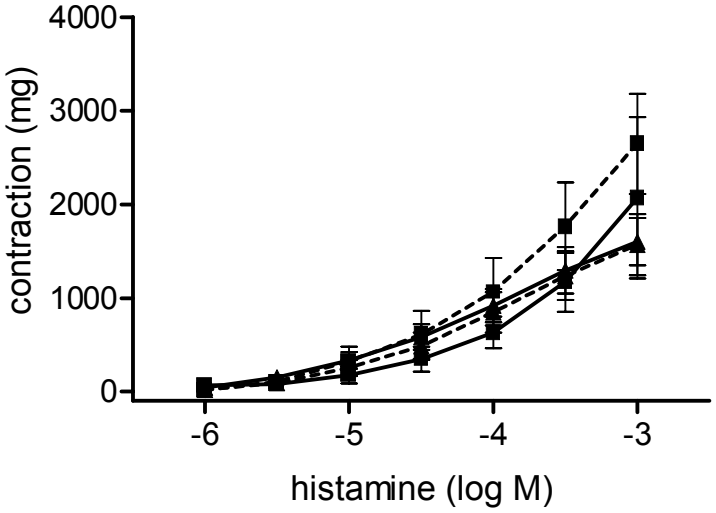

Figure 4. Effect of mucosal inhibition of NO-synthase in guinea pig trachea segments with intact epithelium on contractile responses to mucosal histamine. The NO-synthase inhibitor, L-NAME (dashed lines), tended to increase histamine-induced contractions in proximal (squares), but not in distal (triangles), segments as compared to its inactive enantiomer, D-NAME (solid lines). N = 6 trachea segments per group.

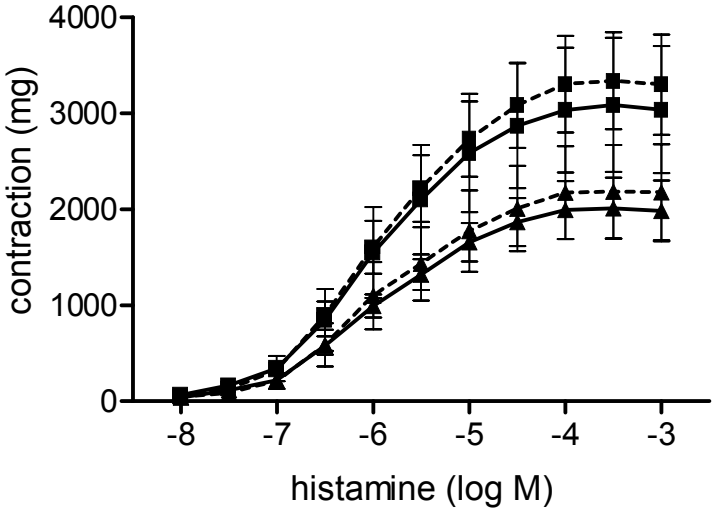

Figure 5. Effect of concomitant mucosal and serosal inhibition of NO-synthase in guinea pig trachea segments without epithelium on contractile responses to mucosal histamine. The NO-synthase inhibitor, L-NAME (dashed lines), had no effect on histamine-induced contractions in proximal (squares) or distal (triangles) segments as compared to its inactive enantiomer, D-NAME (solid lines). N = 6 trachea segments per group.

Since NOS inhibition was without any effect in epithelium-denuded tissues (figure 5), a substantial part of the reactivity differences in epithelium-denuded tracheas is

accounted for by a lack of epithelial NO. However, NO could not completely account for the relaxant activity in proximal segments. Therefore, the contribution of cyclo-oxygenase metabolites to the relaxant activity was studied as well. Like NO, prostaglandins have been shown to counteract histamine-induced contractions in the airways (5, 15). In intact tracheas, cyclo-oxygenase inhibition by indomethacin in the mucosal buffer increased reactivity to histamine in proximal and distal segments to a similar extent (figure 6).

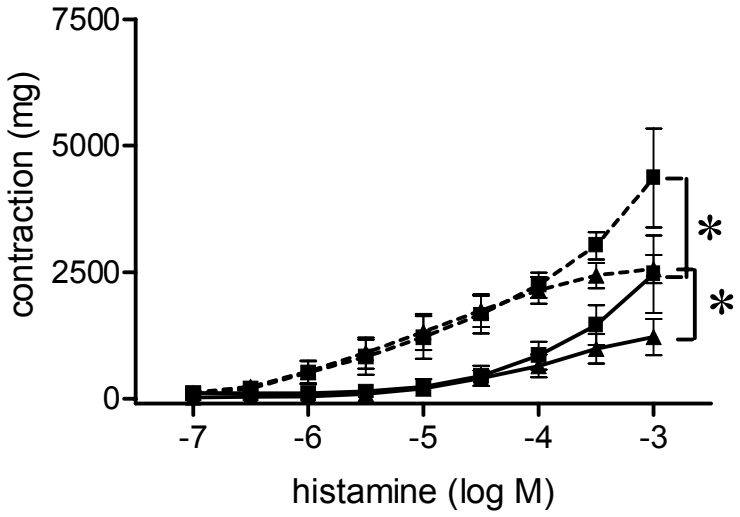

Figure 6. Effect of mucosal cyclo-oxygenase inhibition on contractile responses of guinea pig trachea segments with intact epithelium. Contractions were induced by histamine at the mucosal side. The cyclo-oxygenase inhibitor, indomethacin (dashed lines), increased histamine-induced contractions both in proximal (squares) and in distal segments (triangles) as compared to its vehicle (solid lines). (*, $P < 0.05$) N = 6 trachea segments per group.

This indicates that, contrary to the findings for NO, relaxing cyclo-oxygenase metabolites are synthesized in both proximal and distal trachea segments. Since indomethacin increased tracheal tension when given mucosally, the epithelium is the most likely source of cyclo-oxygenase metabolites in these experiments.

Quite surprisingly, however, indomethacin administered concomitantly at the mucosal and serosal side of epithelium-denuded tracheas led to a marked increase of histamine-induced contractions in proximal, but not in distal, segments as compared to controls (figure 7). The data suggest that in proximal trachea segments, apart from the contributions of epithelium-derived NO, one or more non-epithelial cyclo-oxygenase metabolites clearly compensate for the higher intrinsic smooth muscle reactivity in these segments. This notion was confirmed by the observation that in

proximal segments with intact epithelium, histamine induced stronger contractions when indomethacin was present both in the mucosal and the serosal buffer than

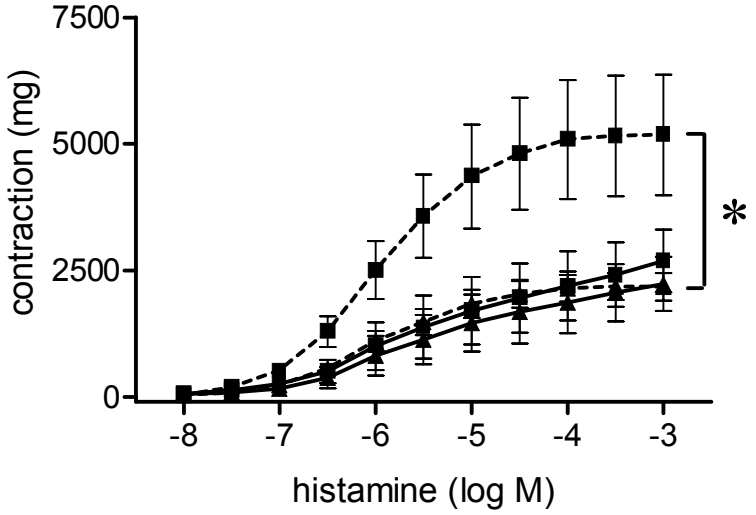

Figure 7. Effect of concomitant serosal and mucosal cyclo-oxygenase inhibition with indomethacin on contractile responses of guinea pig trachea segments without epithelium. Contractions were induced by histamine at the mucosal side. Indomethacin had no effect on histamine-induced contractions in distal segments (dashed line, triangles) compared to its vehicle (solid line, triangles). In contrast, indomethacin substantially increased responsiveness to histamine of proximal segments (dashed line, squares) as compared to vehicle treatment (solid line, squares). (*, $P < 0.05$) N = 6 trachea segments per group.

when indomethacin was administered in the mucosal buffer only (figure 8). Moreover, serosal stimulation with histamine led to an additional increase in tension when mucosal and serosal cyclo-oxygenase was inhibited, but to a decrease in tension when the cyclo-oxygenase inhibitor was only present in the mucosal buffer (figure 8). Thus, in the proximal trachea, cyclo-oxygenase inhibition in serosal tissues has a larger effect on smooth muscle contraction than cyclo-oxygenase inhibition in mucosal tissues. In contrast, inhibition of cyclo-oxygenase in serosal tissues of the distal trachea had no additional effect on smooth muscle reactivity compared to cyclo-oxygenase inhibition in mucosal tissue only (figure 7). Therefore, we conclude that cyclo-oxygenase products from the serosa of the proximal trachea have a dampening effect on trachea smooth muscle reactivity of this segment. This finding may be very relevant. Since histamine induces the production of cyclo-oxygenase products (5, 15) and since histamine is mainly produced by mast cells that inhabit the serosa, serosal cyclo-oxygenase products may be important functional antagonists of allergen-induced contractions in proximal trachea segments.

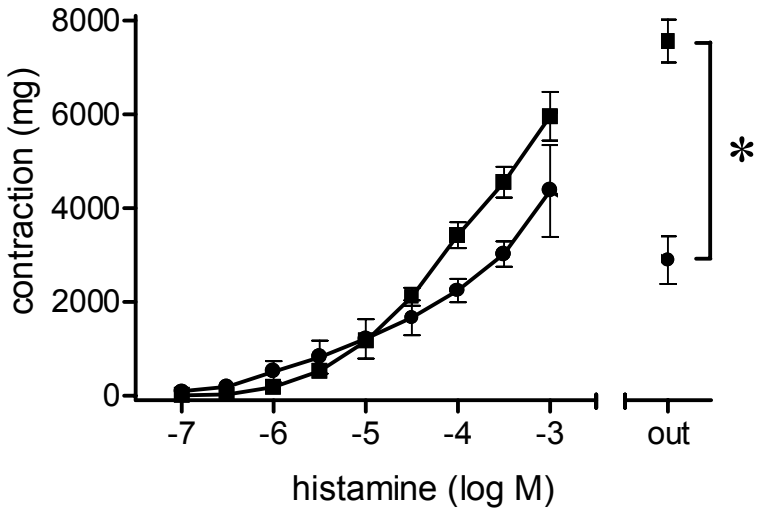

Figure 8. Comparison of the effects of mucosal cyclo-oxygenase inhibition with concomitant mucosal and serosal cyclo-oxygenase inhibition in proximal trachea segments with intact epithelium. Mucosal histamine induced stronger contractions when the cyclo-oxygenase inhibitor, indomethacin, was present both in the mucosal and the serosal buffer (squares) than when indomethacin was administered in the mucosal buffer only (circles). Histamine given serosally (out) after the mucosal doses led to a decrease in tension in the absence of a cyclo-oxygenase inhibitor in the serosal buffer, but to an additional increase in tension when a cyclo-oxygenase inhibitor was present in the serosal buffer. (*, $P < 0.05$ (with *vs.* without serosal cyclo-oxygenase inhibitor)) N = 9 trachea segments per group.

A substance of which we recently showed that it could moderate airway tension (chapters 3, 4, 5, 6) is GSH. Since levels of both GSH and its oxidized form, GSSG, were similar in proximal and distal trachea segments (Table 1), it is excluded that the differential reactivity can be accounted for by different levels in proximal and distal trachea of this thiol.

	proximal	distal
GSH	485 ± 22.3	465 ± 49.4
GSSG	6.5 ± 3.7	10.1 ± 5.4

Table 1. GSH and GSSG content (nmol/g wet tissue) of proximal and distal trachea segments are not significantly different. Figures represent the mean ± SEM of three samples, each containing 3 segments.

The introduction of the perfused trachea organ bath system made it possible to study the consequences of selective stimulation of the mucosal or serosal tissues. Such studies have identified the tracheal epithelium as a source of relaxing factors (see (16) for a review). However, to our knowledge, this is the first study to demonstrate that the serosa has a distinct role in the modulation of tracheal tone as well and that this is particularly true for the proximal part of the trachea. Further research is needed to identify which cyclo-oxygenase products counteract contraction and which cells produce the cyclo-oxygenase products in the serosa. In addition to the epithelium, constitutive cyclo-oxygenase expression has been demonstrated in cultured human airway fibroblasts and smooth muscle cells (17).

Another question that remains to be answered is whether hyperreactivity induced by cyclo-oxygenase inhibition is indeed merely the result of downregulating the synthesis of cyclo-oxygenase products. Since cyclo-oxygenase and lipoxygenase compete for arachidonic acid, cyclo-oxygenase inhibition may increase the synthesis of lipoxygenase products (18). This may provide an alternative mechanism for indomethacin-induced hyperreactivity in these experiments. To test this, experiments in the presence of leukotriene receptor antagonists, or the combined presence of cyclo-oxygenase and lipoxygenase inhibitors, should be done.

We conclude that in the guinea pig, intrinsic contractility of tracheal smooth muscle tissue is higher in the proximal than in the distal segment. This higher contractility is clearly compensated for both by mucosal NO and serosal cyclo-oxygenase products. Neglecting these differences can easily lead to larger variances when studying multiple pieces of one trachea. This may lead to the use of more, rather than less experimental animals in order to attain statistically significant results. In addition, false conclusions may be drawn regarding intrinsic smooth muscle contractility, or the importance of NOS or cyclo-oxygenase in the trachea. Conceivably, similar complications may apply to other pathways as well. Thus, it is important to perform pilot experiments to investigate whether there are differences within the trachea regarding the effects on tracheal tone of the particular pathway under study.

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3

Glutathione and other low-molecular-weight thiols relax guinea pig trachea *ex vivo* – interactions with nitric oxide?

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Abstract

The aim of this study was to determine the effects of glutathione (GSH) on trachea smooth muscle tension in view of previously reported interactions between GSH and nitric oxide (NO), and the high (millimolar) concentrations of GSH in trachea epithelium.

GSH and other thiols (1.0 – 10 mM) dose-dependently decreased the tension in isolated epithelium-denuded guinea pig tracheas, and less so in intact tracheas. Relaxations by GSH were paralleled by a 7-fold increase in nitrite levels ($P < 0.05$) in the tracheal effluent, suggesting an interaction between GSH and NO. However, preincubation with the NO scavenger, PTIO (100 μM), did not reduce the relaxations by GSH or its NO-adduct, S-nitrosoglutathione (GSNO).

Inhibition of guanylyl cyclase with ODQ (50 μM) inhibited the relaxations induced by GSNO, but not by GSH. Blocking potassium channels with tetraethylammonium (20 mM), however, completely abolished the relaxing effects of GSH ($P < 0.05$).

Preincubation of tracheas with GSH significantly ($P < 0.05$) suppressed hyperreactivity to histamine as caused by removal of tracheal epithelium. In contrast, GSH did not significantly affect histamine-induced contractions in intact tracheas. These data indicate that GSH plays a role in maintaining tracheal tone. The mechanism behind this is possibly an anti-oxidative action of GSH itself, rather than an action of NO or GSNO.

Introduction

Airway hyperresponsiveness is a key feature of several lung diseases. It is often associated with epithelial damage as a consequence of inflammatory processes (1). Damaged epithelium is impeded in its role of protecting the underlying smooth muscle against contractile stimuli. Moreover, the function of the epithelium as a source for relaxing factors that compensate for contractile stimuli will be impaired (2). One of those endogenous airway smooth muscle relaxants is nitric oxide (NO). NO is produced by a variety of cells and tissues in the respiratory tract, including the epithelial layer (3, 4). Under physiological conditions, genuine NO is very unstable and rapidly loses its biological activity by reacting almost instantaneously with oxygen, superoxide anion and transition metals (5-7). Maintenance of an appropriate smooth muscle tone in the airways, therefore, requires continuous synthesis as well as stabilization of NO. Thiols are excellent candidates for the latter purpose. Under aerobic conditions, NO reacts with thiols to form nitrosothiols (RSNOs) via the nitrosylating intermediate dinitrogen trioxide (8). RSNOs are also produced by direct binding of nitrosonium ions to thiols (9). RSNOs can be regarded as stable pools of NO (10). Whereas in principle any thiol can bind NO, glutathione (GSH) is probably especially important in this respect. GSH is the major representative of the class of non-protein thiols and plays a pivotal role in a variety of enzymatic and non-enzymatic reactions that protect tissues against oxidative stress (11). In view of the anti-oxidant role of GSH and wide spread interactions between oxygen and tissues in the airways, it is not surprising that the airways are among the tissues containing the highest GSH concentrations in the body. Especially lung epithelial cells (12) and the epithelial lining fluid in the lungs contain high levels of GSH (13).

In anti-oxidative 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. Under normal conditions, 95 – 99% of total glutathione in the body is present in the reduced form (11). However, inflammatory diseases like asthma are associated with oxidative stress that places a large burden on the GSH pool. This may result in decreased levels of GSH available for NO stabilization and thus contribute to the development of airway hyperresponsiveness. Indeed, evidence was recently presented that an oxidative imbalance in the airways of asthmatics is reflected, among other parameters, by high levels of GSSG (14).
To address these issues, we tested whether addition of GSH to epithelium-denuded guinea pig tracheas increased NO levels as judged by a rise of nitrite levels in the perfusion buffer and, if so, whether the increased nitrite levels showed a causal

correlation with relaxation of tracheal smooth muscle. Since GSH-induced relaxations were indeed paralleled by a rise in nitrite levels in the buffer, it was investigated whether these relaxations were mediated by guanylyl cyclase or by potassium channels, both of which are known play a role in NO-induced smooth muscle relaxation. Finally, the physiologic relevance of alteration of tracheal tension by GSH was assessed by measuring whether perfusion of tracheas with GSH could moderate the hyperresponsiveness resulting from removal of their epithelium.

Methods

Animals and organ bath experiments

Male specific pathogen-free Dunkin Hartley guinea pigs weighing 350 - 400 g (Harlan Nederland, Horst, The Netherlands) were housed under controlled conditions. Water and commercial chow were allowed *ad libitum*. Guinea pigs were killed with an overdose of pentobarbitone-sodium (Nembutal®, 0.6 g/kg body weight, *i.p.*). Tracheas were dissected free of connective tissue and blood vessels, isolated, and divided into a proximal and distal part. Where indicated, the epithelial layer was removed from the tracheal segments as described earlier (15). Tracheas were mounted in perfused organ baths according to a modified method of (16). The organ baths contained Krebs buffer of the following composition (mM): NaCl (118.1), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaHCO_3$ (25.0), K_2HPO_4 (1.2), glucose (8.3). The lumen of the trachea was perfused with Krebs solution independently from the outside by means of a peristaltic pump delivering a flow rate of 2 ml/min. The Krebs solution was continuously gassed with 5% CO_2 in O_2 and kept at 37°C. Two steel hooks were inserted through opposite sites of the tracheal wall with the smooth muscle between them. The lower hook was fixed to the bottom of the organ bath; the other hook was attached to an isometric force transducer (Harvard Bioscience, Kent, UK). Transducers were connected to an analogue-digital converter, delivering digital signals to a computerized set-up. The setup allowed continuous sampling, on-line equilibrium detection and real-time display of the responses on a computer screen.

The tracheal tension was set at an optimum counter weight of 4.0 or 2.0 g for thiol-induced relaxations and histamine-induced contractions, respectively. The use of different pretensions for assessing effects of relaxing and contractile agents is common practice in organ bath studies. The tissues were allowed to reach a stable tone for 60 min, during which the buffer was refreshed every 15 min. If necessary, tissues were allowed additional time to equilibrate without the buffer solution being changed.

Thiol-induced tracheal relaxation

Epithelium-denuded tracheas were consecutively perfused with a range of concentrations (0.1 to 10 mM) of GSH, L-cysteine (Cys) or N-acetyl-L-cysteine (NAC). As a control, the non-thiol amino acid L-valine was tested at the same concentrations. In a separate set of experiments, GSH-induced relaxations were recorded in epithelium-denuded and in intact tracheal tubes, using the same range of concentrations as mentioned above.

Mechanisms of GSH-induced tracheal relaxations

Interactions with NO

For NO measurements, nitrite was assayed as a stable and representative breakdown product of NO formed enzymatically or NO released from nitrosothiols (9). Samples of 100 µl tracheal effluent were collected just prior to or immediately after addition of GSH. The samples were injected into a purge vessel containing 2 ml of a 1% solution of sodium iodide in glacial acetic acid. The purge vessel was connected to a Sievers 270B NO analyzer (Boulder, CO, USA). The sensitivity of the NO analyzer is 10 pmol/ml with a linearity of four log orders of magnitude. Calibrations were made according to the manufacturer's instructions with standard solutions of sodium nitrite (17).

In a separate set of experiments, the potential role of free NO in GSH-induced relaxations was investigated using the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (18). PTIO was added to the luminal buffer after the 60-minute-equilibrium period at a final concentration of 100 µM. Twenty min after adding PTIO, tracheas were relaxed by intraluminal addition of GSH, GSNO or GTN. The compounds were given in concentrations that evoke a sub-maximal response, i.e. 5.0 mM, 100 µM and 10 µM for GSH, GSNO and GTN, respectively. PTIO remained in the buffer during the relaxations.

The inhibitor of soluble guanylyl cyclase (sGC), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (50 µM) (19), or the non-selective potassium (K^+-) channel blocker, tetraethylammonium (TEA) (20 mM) (20), were applied both to the mucosal and the serosal buffer. Controls received the vehicle of ODQ (DMSO; 0.25% (v/v) final concentration). After 20 min of incubation, GSH (5 mM) or S-nitrosoglutathione (GSNO, 100 µM) was added to the luminal buffer without removing TEA or ODQ and relaxations were recorded.

Interactions with PGE_2

A 100 µl sample of the luminal perfusate of tracheas was taken before and after the addition of GSH to be assayed for PGE_2 content. PGE_2 was quantified with an

enzyme-linked immunoassay (Amersham, Roosendaal, The Netherlands) according to manufacturer's instructions.

Effect of GSH on tracheal responsiveness to histamine
Epithelium-denuded tracheas were pre-incubated intraluminally with GSH at a concentration of 1.0 mM, or saline (controls). After 40 min, optimum tension (2.0 g) was re-adjusted mechanically and contractions were measured to increasing concentrations (10^{-8} to 10^{-3} M) of histamine in the inside buffer. GSH or saline were left in the buffer during histamine-induced contractions.

Drugs
GSH, NAC, Cys and L-valine were obtained from Sigma, St. Louis, MO. ODQ, GSNO and PTIO were purchased from Alexis, Lausen, Switzerland and TEA from Merck, Darmstadt, Germany. Glyceryl trinitrate (GTN) was obtained from Brocacef, Maarssen, The Netherlands.

Data analysis
Relaxations were determined as the percentage of the 4.0 g baseline tone that was set after completion of the equilibration period. Contractions were expressed as mg tension on top of the 2.0 g baseline tone. Data are expressed as mean ± SEM. For most experiments, significance calculations were performed using the two-tailed Student's t-test. However, the Wilcoxon signed-rank test was used for assessing the statistics of the nitrite measurements, while a repeated measures analysis with L.S.D. post hoc test was used for analysis of the data pertaining to histamine reactivity. Differences were considered statistically significant if $P < 0.05$.

Results

Thiol-induced tracheal relaxation
GSH, as well as the sulfhydryl amino acids, Cys and NAC, relaxed tracheas without epithelium dose-dependently at concentrations of 1.0 mM and higher (figure 1A). Relaxations started within seconds after administration of thiols (figure 2 shows a representative tracing of a relaxant response to GSH; the profile of the relaxations was similar for all thiols). The sensitivity to the tested compounds increased in the order Cys << GSH < NAC. When administered at the highest concentration (10 mM), Cys, GSH and NAC reduced the initial 4.0 g baseline tension by about 20, 65 and 70%, respectively. As expected, L-valine, which does not have a thiol group, did not significantly alter baseline tension at any concentration (data not shown).

GSH-induced relaxations were more extensive in denuded tissues than in tissues with intact epithelium (figure 1B). At 10 mM, GSH reduced the initial 4.0 g baseline tension by 65.7 ± 6.5% in denuded tracheas and by 36.7 ± 5.9% in intact tracheal tubes.

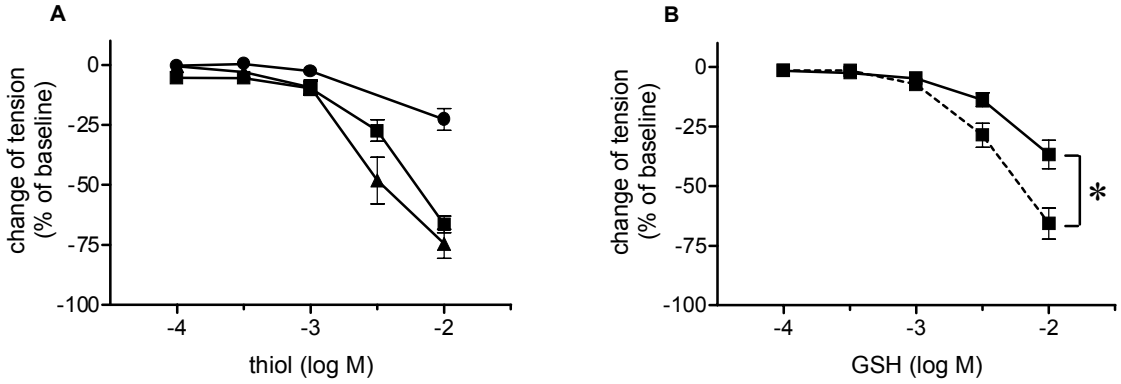

Figure 1. Relaxing effects of the thiol compounds, GSH (squares), NAC (triangles) and Cys (circles) in guinea pig tracheas without epithelium (A) and the inhibitory influence of the epithelium on the relaxing effects of GSH (B; solid line, intact trachea; dashed line, denuded trachea). Change of tension is expressed as the percentage of a 4.0 g baseline tone. * $P < 0.05$; $N = 9$ in each group.

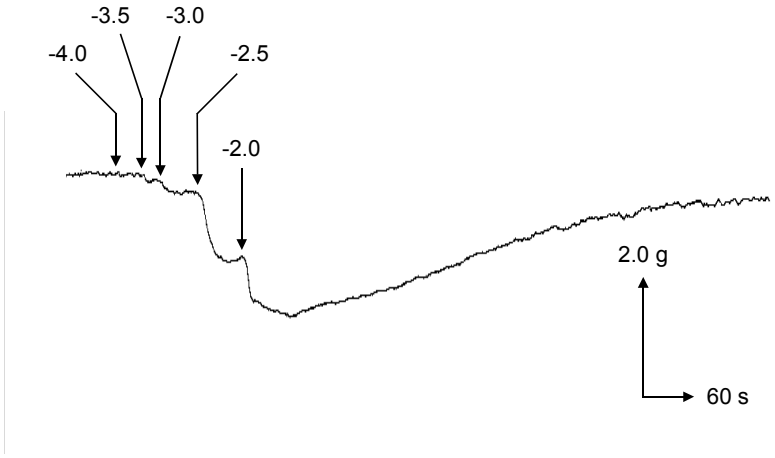

Figure 2. Representative tracing of the relaxation of an epithelium-denuded tracheal tube as induced by increasing concentrations of GSH in the luminal buffer. Arrows indicate time of administration; the respective GSH concentrations (log molar) are indicated.

Mechanisms of GSH-induced tracheal relaxations

Since NO is a well-known smooth muscle relaxant that can interact with thiols, we measured whether perfusion with GSH increased nitrite levels in the tracheal effluent.

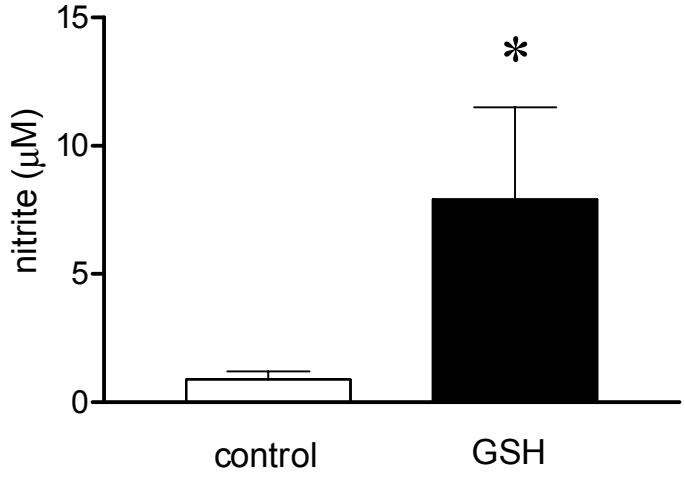

Figure 3. Effect of addition of GSH on nitrite concentrations in the luminal buffer. Samples were taken just before (control) and immediately after addition of GSH (10 mM, final concentration) to the perfusing buffer. * $P < 0.05$; N = 6 in each group.

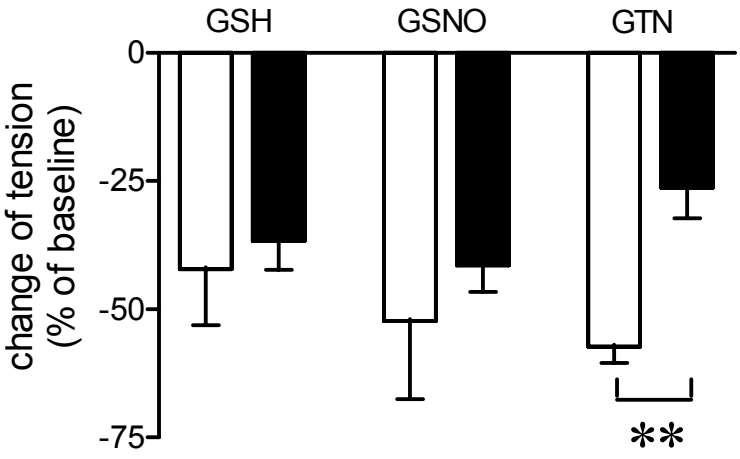

Figure 4. Effect of scavenging free NO by PTIO (100 μM), on relaxations induced by GSH, GSNO or GTN in isolated perfused guinea pig trachea. White bars, control; black bars, PTIO. Change of tension is expressed as the percentage of a 4.0 g baseline tone. ** $P < 0.01$; N = 6 in each group.

After the 60-minute-stabilization period, *i.e.* just before addition of GSH, nitrite levels in the tracheal effluent were 0.9 (95% confidence interval, 0.62 – 1.2) μM, whereas immediately after addition of 10 mM GSH, levels increased significantly ($P < 0.05$) to 7.9 (95% confidence interval 4.3 – 11.4) μM (figure 3), suggesting release of NO from the tissue by GSH.

To further address a possible role for NO in GSH-induced relaxations, the NO-scavenger, PTIO (18), was added to the perfusion buffer prior to administration of GSH, GSNO or GTN. PTIO did not significantly alter GSH- or GSNO-induced relaxations (figure 4). Since PTIO, in contrast, significantly decreased relaxations by the genuine NO donor, GTN, it is unlikely that the GSH- and GSNO-induced relaxations were mediated through free extracellular NO. Furthermore, it was investigated whether a major target of NO and GSNO, guanylyl cyclase, was involved in the relaxations evoked by GSH. Preincubation with the sGC inhibitor, ODQ (21), did not affect the ability of GSH to cause tracheal relaxation (figure 5), suggesting that the relaxing effect of GSH was not due to a rise in cGMP levels.

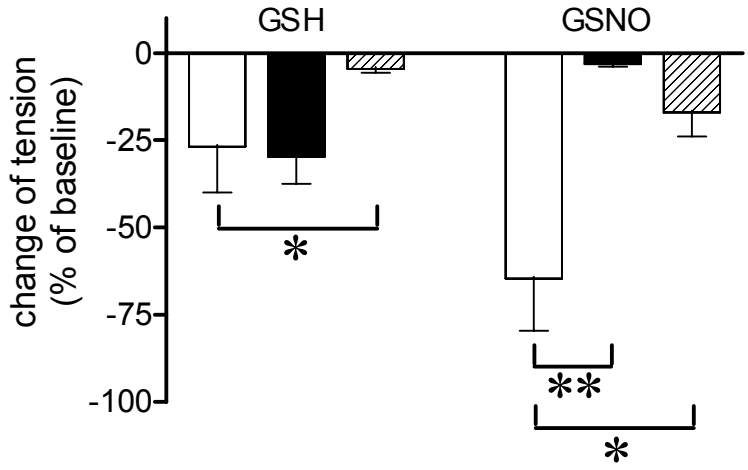

Figure 5. Effects of inhibition of sGC (ODQ, 50 μM) or K^+-channels (TEA, 20 mM) on GSH-induced (5 mM) relaxation of isolated and perfused guinea pig tracheas. For comparison, another set of tracheas received GSNO instead of GSH. White bars, control; black bars, ODQ; hatched bars, TEA. Change of tension is expressed as the percentage of a 4.0 g baseline tone.
* $P < 0.05$, ** $P < 0.01$; $N = 6$ in each group.

An alternative target in smooth muscle relaxation by NO are K^+-channels. Upon treatment with the non-specific K^+-channel inhibitor TEA (20), the GSH-induced relaxations were almost completely abolished (figure 5), suggesting that K^+-channels mediate thiol-induced relaxations. Further, it was investigated whether

GSNO, a potential product of interaction of GSH and NO, mediates GSH-induced relaxations. To this end, the experiments with ODQ and TEA were carried out with GSNO as the relaxing agent. In these experiments, relaxations were inhibited both by ODQ and, to a lesser extent, TEA (figure 5).

Interactions with PGE_2

Levels of PGE_2 in the luminal perfusate were 134 ± 18 pg/ml under baseline conditions. Upon addition of GSH, PGE_2 levels in the perfusate remained unchanged (124 ± 57 pg/ml; N = 6).

Effect of GSH on tracheal responsiveness to histamine

Perfusion of intact tracheas with histamine caused a moderate concentration-dependent increase of smooth muscle tension, whereas contraction after removal of epithelium started at lower concentrations of histamine and was markedly stronger.

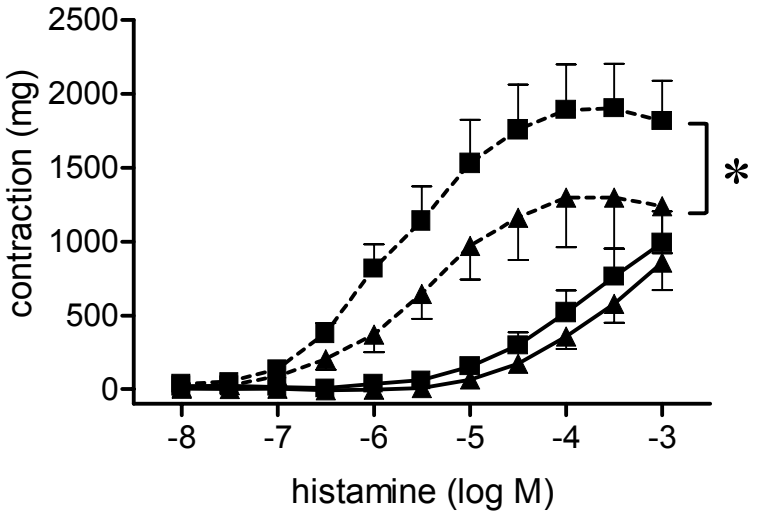

Figure 6. Effect of GSH on histamine-induced contractions in isolated perfused tracheas. Intact and epithelium-denuded tracheas were exposed to GSH (1.0 mM) before and during histamine-induced contractions. Squares, control; triangles, GSH; solid lines, intact trachea; dashed lines, denuded trachea. * $P < 0.05$; N = 9 per group.

This hyperresponsiveness upon removal of epithelium has been reported earlier (22, 23). Preincubation with 1.0 mM GSH significantly attenuated the hyperreactivity in tracheas without epithelium by 31.6 ± 9.46 % in terms of maximum response to histamine, but did not change responsiveness of tracheas with intact epithelium (figure 6).

Discussion

In this study, we have shown that GSH and other thiols reduced the smooth muscle tone of epithelium-denuded guinea pig tracheas in perfused organ baths. Relaxations up to 65 to 70% below baseline tension were induced by 10 mM GSH or NAC, the highest concentration tested (figure 1A). This concentration may be physiologically relevant since GSH is estimated to be present at this concentration in epithelial cells (12). Relaxations induced by Cys appeared less pronounced. Whether this indicates that the thiols need to be taken up in target cells to exert their relaxing effect is not clear. NAC, but not Cys, diffuses readily across the cell membrane. Uptake of GSH, however, has been shown to require an active transport mechanism that is present in epithelial cells, but absent in many other cell types tested (24-26). In addition, the almost instantaneous relaxation induced by the thiols points to an extracellular rather than an intracellular mechanism. L-valine failed to relax tracheas at any concentration, suggesting that induction of relaxation requires a sulfhydryl group and was not due to non-specific effects such as osmolarity changes in the perfusion buffer. GSSG may be better than L-valine to control for these properties, but we obtained inconsistent results with GSSG. The compound had no effect on most tracheas tested (N = 6), but it relaxed some tracheas (N = 3) to the same extent as GSH. The latter observation is hard to explain. Since relatively high concentrations were required for relaxation, instantaneous reduction of GSSG to GSH by the latter tracheas seems unlikely.
Relaxations were more pronounced in epithelium-denuded tracheas, than in intact tissues (figure 1B). The epithelium, therefore, possibly forms a physical barrier against relaxation by GSH on the underlying smooth muscle. Alternatively, intact epithelium already reduces tracheal tension by supplying the smooth muscle layer with GSH, so that additional GSH can only have a limited effect.

Interestingly, GSH-induced relaxations in epithelium-denuded tissues were paralleled by a seven-fold rise in nitrite levels in the tracheal effluent (figure 3), suggesting release of NO by GSH (9). In view of the absence of the epithelium, the increase of nitrite levels has to be derived from other sources than the NO-rich epithelial cells. Sensory nerve endings in the trachea might have provided the NO and thus be the putative source of nitrite (27). It is doubtful, however, whether GSH caused relaxation by releasing genuine NO, in view of the effect of the free NO scavenger, PTIO (18). This agent failed to inhibit relaxation by GSH while it clearly inhibited relaxation by the genuine NO donor, GTN (28) (figure 4). So, free NO, whether or not produced by nerve endings in the epithelium-denuded tracheas, is an unlikely mediator of the GSH-induced relaxation and nitrite formation.

Alternatively, the GSH effects may involve nitrosylated proteins and other molecules in the sub-epithelial tissues. Those are likely to have been formed there prior to removal of the epithelium, because NO once produced can diffuse to neighboring cells and nitrosylate protein and non-protein thiols via nitrosylating agents, like dinitrogen trioxide (8) and nitrosonium ions (9). GSH would then interact with tissue RSNOs to yield nitrite without the appearance of NO as an intermediate (29). It is also known that GSH forms GSNO in the presence of protein RSNOs (30). Although GSNO can cause tracheal relaxation, this molecule is also unlikely to be the mediator of the GSH-induced relaxations, since inhibition by ODQ of sGC, the primary target of NO and RSNOs (19), abolished the relaxing effect of exogenous GSNO, but not of GSH.
To check whether PGE_2 could mediate the GSH-induced relaxations, this major relaxant prostanoid was measured in the perfusate of epithelium-denuded tracheas before and during the GSH-induced relaxation. PGE_2 levels remained unaltered upon addition of GSH to the organ bath buffer. These data show that PGE_2 is not mediating GSH-induced relaxation.

In a further attempt to find a target of GSH that mediated the relaxations, the effects of the non-specific K^+-channel inhibitor TEA were investigated. TEA almost completely abolished trachea relaxation by GSH (figure 5), suggesting that GSH can activate particular K^+-channels in this tissue. It is not unlikely that this is due to modification of sulfhydryl groups, since sulfhydryl reagents and other oxidizing compounds were reported to inactivate various K^+-channels, while sulfhydryl reducing agents, like dithiothreitol and GSH, were shown to reverse inactivation or to cause their activation (31, 32). Further studies are needed identify which K^+-channel is involved in the observed relaxation and whether activation of that channel is regulated by sulfhydryl modification.

Interestingly, K^+-channel inhibition also inhibited GSNO-induced trachea relaxation. Hence, in guinea pig trachea, GSNO apparently induces relaxation through cGMP-induced K^+-channel activation. cGMP-dependent activation of calcium-activated K^+-channels by nitrosothiols has been reported earlier in rabbit coronary artery smooth muscle (33).

GSH not only caused trachea relaxation, but also counteracted histamine-induced contraction in epithelium-denuded, but not intact, tracheas (figure 6). The more pronounced activity in epithelium-free tracheas is probably due to the same mechanisms as pointed out above. The finding, however, is relevant since airway hyperresponsiveness is associated with sloughing of the epithelial layer (1).

Smooth muscle cell relaxation by GSH and other reduced thiols may represent a novel mechanism to maintain tracheal tone. Interestingly, this mechanism is probably not mediated by NO or GSNO, but possibly by an anti-oxidative action of GSH and other thiol compounds in the airways. Furthermore, the capacity of GSH to attenuate hyperreactivity in a model for damaged epithelium such as occurs in asthma (Figure 6) indicates that replenishment of GSH in the airways may have therapeutic potential at physiological concentrations. Thiol replenishment in asthma has only been described once. Nebulization of GSH in mild asthmatics caused bronchoconstriction rather than a relief of symptoms (34), but this adverse effect may have been caused by the supra-physiological concentration of GSH (0.5 M), which was 500 times higher than the concentrations we used to dampen histamine-induced contractions. In a proper dose, replenishing the sulfhydryl content in the airways could be a potential therapy in diseases where excessive bronchoconstriction and oxidative stress are concomitant features.

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4

Acute glutathione depletion underlies the early asthmatic response in a guinea pig model of allergic asthma

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Abstract

Substantial evidence suggests that oxidative stress in the lungs can affect the pathological events observed in asthma. 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 a decrease in 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*. Glutathione-ethyl ester, 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 modulates airway responsiveness in the early asthmatic reaction.

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).
The antioxidant, glutathione (GSH) is present in 15 mM concentrations in cultured human lung epithelial cells (7) which is considerably higher than the concentrations reported for most other cells (8). GSH concentrations in lung epithelial lining fluid *in vivo* are approximately 450 μM; this is 100-fold higher than in plasma (9).
As an antioxidant, GSH is converted into its homodimeric disulfide, oxidized glutathione (GSSG) (8). Increased formation of reactive oxygen species, resulting from inflammation, enhances formation of GSSG and decreases the tissue content of GSH (10). Although decreased GSH levels have been found in broncho-alveolar lavage (BAL) fluid in several lung diseases (11), the BAL fluid from stable asthmatics contains higher total glutathione levels than normal subjects (12). This increase is thought to reflect an adaptive response to the oxidant stress of inflammation (13).

GSH is known to influence rabbit and rat vascular (14, 15) and pig gastric (16) smooth muscle relaxation. We recently 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 (chapter 3). 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 first 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.

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,

$Al(OH)_3$ (Merck, Darmstadt, Germany), in 1.0 ml saline. $Al(OH)_3$ 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.

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) 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 (17). BSO was dissolved in PBS and 2.5 mmol/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 (18). 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 37ºC, 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, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.05 mM, KCl 2.68 mM, $NaHCO_3$ 0.6 mM, NaH_2PO_4 0.13 mM, and Na_2HPO_4 0.896 mM. The buffer was warmed to 45ºC and pumped at a rate of 2 ml/min through a bubble trap before being cooled to 37ºC 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 (18). 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.

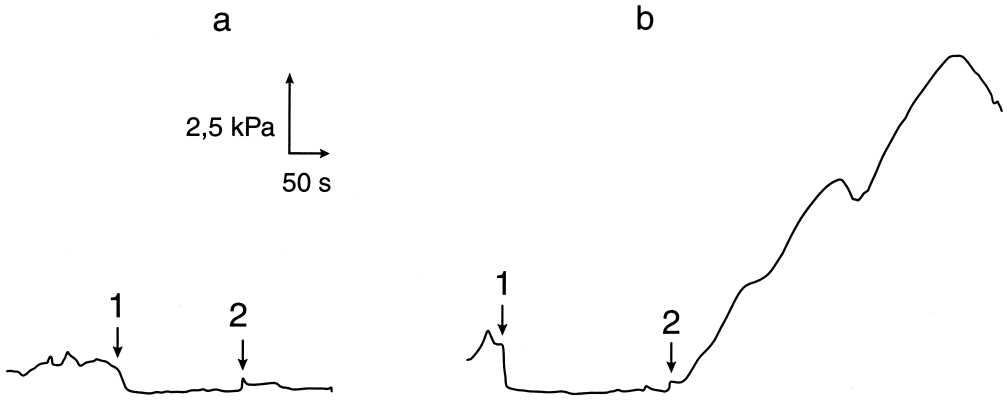

Figure 1. Representative tracings of the airway opening pressure (Pao) during an allergen-induced early asthmatic reaction (EAR). Lungs from sham- (**a**) or OVA- (**b**) sensitized guinea pigs were challenged ex vivo with OVA in the perfusion buffer. The EAR-induced contraction of the lungs increased 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 airway contraction.

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 (figure 1). 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^{-9} - 10^{-5} 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
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 $HClO_4$ 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, the tubes were centrifuged at 5000 *g* for 10 min and total and oxidized glutathione concentrations in the supernatants were determined with a modified version (19) of the glutathione reductase-DTNB recycling assay according to Akerboom *et al*. (20). 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_{max}) were tested for significance. Differences were considered statistically significant if $P < 0.05$.

Results

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 (figure 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 ($P < 0.0001$).

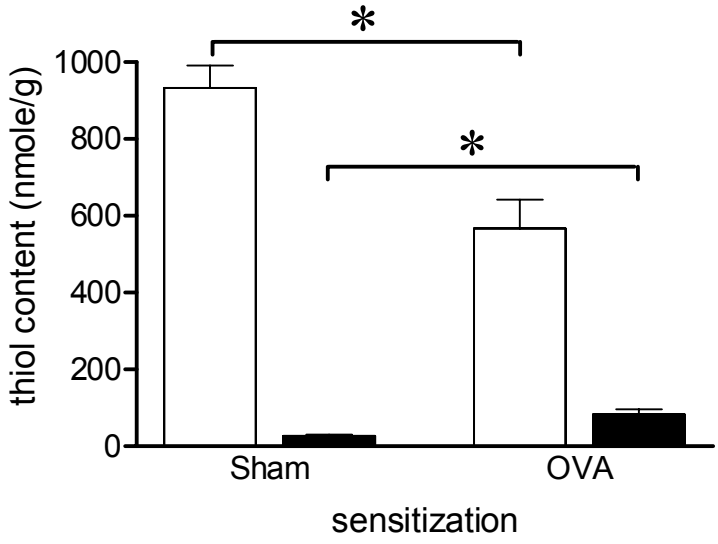

Figure 2. Effect of an EAR on lung glutathione levels. Sham- or OVA-sensitized guinea pigs were challenged with an OVA aerosol, after which lungs were harvested to measure GSH and GSSG. Compared to lungs of sham-sensitized animals, lungs from OVA-sensitized guinea pigs contained less GSH (white bars), while GSSG levels (black bars) were increased.
Data are expressed as means ± SEM (N = 8); * $P < 0.005$

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 (figure 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. 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).

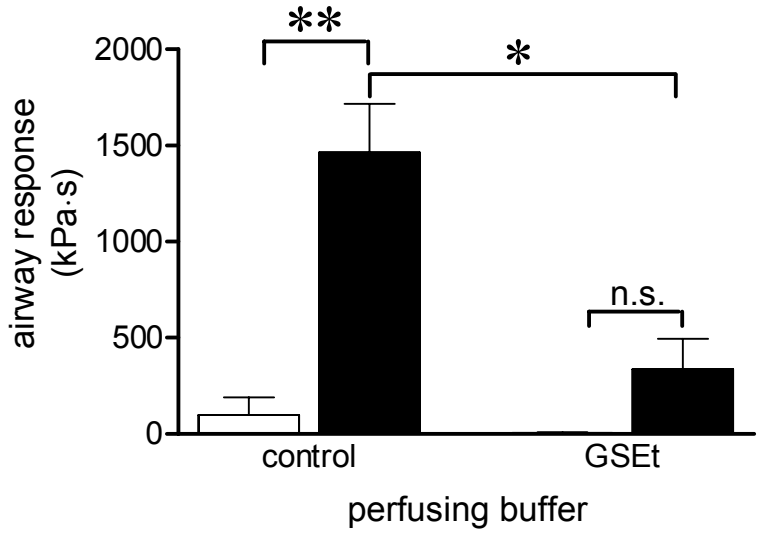

Figure 3. Effect of an EAR on airway opening pressure (Pao) in the absence or presence of the GSH donor, GSEt, in the perfusing buffer. Conditions of the experiment are the same as described for figure 1. White bars, sham-sensitized animals; black bars, OVA-sensitized animals.
Data are expressed as means ± SEM (N = 7); * $P < 0.01$; **, $P < 0.005$

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% (figure 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 (figure 4b). Since in the GSEt treated group the differences in GSH and GSSG levels between BSO-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±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 (figure 5a), reaching a maximum Pao of 13.0±1.3 kPa ($P < 0.05$). GSEt in the perfusing buffer prevented BSO-induced increases in histamine responsiveness (figure 5b).

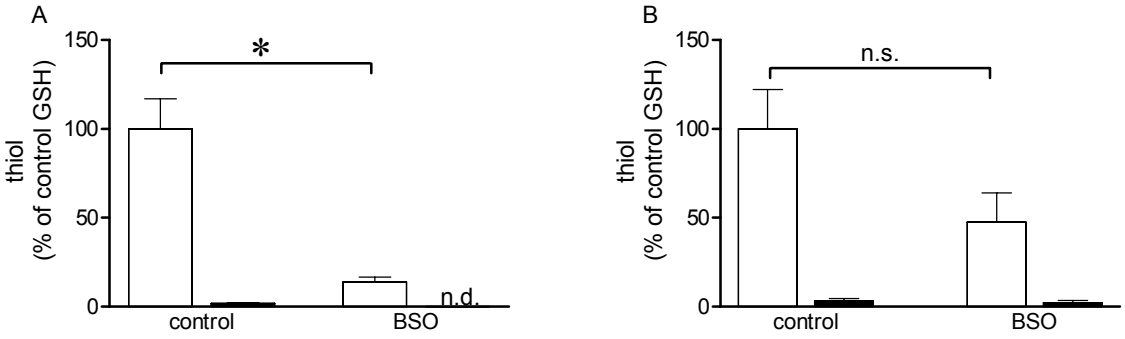

Figure 4. Effect of BSO treatment and GSEt perfusion on lung glutathione levels. A. BSO treatment decreased guinea pig lung GSH levels (white bars; N = 10) by 85% compared to control animals (N = 8), while GSSG (black bars) in BSO-treated animals could no longer be detected. B. Perfusion with GSEt partially restored lung GSH and GSSG levels (N = 7). Data are expressed as means ± SEM. n.d., not detectable; n.s., not significantly different; * $P < 0.005$

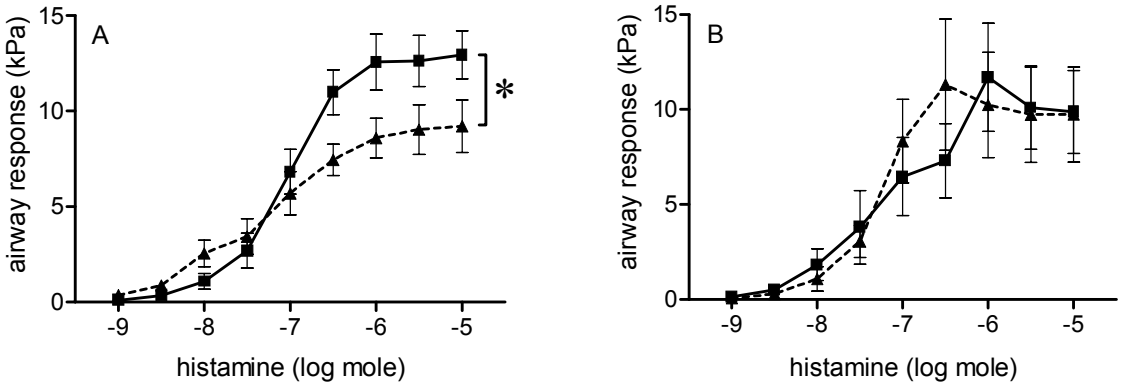

Figure 5. A. Effect of GSH depletion on histamine reactivity of isolated lungs. Histamine-induced contractions in isolated lungs of GSH-depleted guinea pigs were higher than those in lungs of control animals. Data are expressed as means ± SEM (BSO, N = 10; control, N = 8). Triangles, controls; squares, BSO-treated animals. * $P < 0.05$. B. Effect of supplementary GSH on histamine responses of GSH-depleted guinea pig lungs. Perfusion with the GSH donor, GSEt (1 mM), prevented hyperresponsiveness of lungs depleted of GSH by prior BSO treatment. Data are expressed as means ± SEM (N = 7, both groups). Triangles, controls; squares, BSO-treated animals.

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; the latter effect could be reversed by addition of GSEt.

The occurrence of oxidative stress during the primary EAR is a remarkable finding, since inflammatory cells have not yet been recruited into 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* (21). Moreover, histamine release from cultured rat mast cells appeared to require oxidation of arachidonic acid by oxygen-centered free radicals (22). Allergen-induced mast cell 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. Interestingly, it was shown that the formation of histamine-releasing substances from arachidonic acid by oxygen-centered free radicals can be inhibited by several free radical scavengers, including GSH (22). This is in line with our findings that a cell permeable GSH-precursor inhibited airway contraction during the EAR. The identity of the oxidative arachidonic acid products is obscure. A candidate that has gained increasing attention in the field of oxidative stress markers is 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$); the latter product is a mainly non-cyclo-oxygenase-derived prostaglandin that is abundantly produced during oxidative stress (23). 8-*Iso*-$PGF_{2\alpha}$ is an airway smooth muscle contractant (24) and was found to be elevated in plasma (25) and exhaled breath condensate (26) of asthmatics. Hence, 8-*iso*-$PGF_{2\alpha}$ is possibly involved in the EAR and this is subject of our current investigations. Airway responses to the contractile agent that was studied in the current paper, histamine, were 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 also influence relaxant substances. GSH is known to enhance the bioavailability of nitric oxide (NO) (27), a major airway smooth muscle relaxant that, moreover, antagonizes histamine release by mast cells (28). Thus, depletion of GSH may increase airway contractility by hampering the effects of NO. Another airway smooth muscle relaxant is PGE_2. PGE_2 synthesis was found to be downregulated in renal homogenates by limiting GSH concentrations (29). 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 showing that total glutathione levels in lung lavage fluid are higher in stable asthmatics than in healthy subjects (12) 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 (17), including cultured human lung epithelial cells (30). Thus, the enhanced GSH levels in stable asthmatics are likely to be the result of GSH depletion by the local inflammatory cells and possibly by preceding exacerbations.

This paper has shown that airway GSH levels in an animal model of an EAR are acutely decreased. Furthermore, decreased GSH is associated with enhanced airway responses. This could be due to interference of GSH with different mechanisms, in particular an 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 approach for innovative therapy.

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5

A whey-based glutathione enhancing diet decreases allergen-induced airway contraction in a guinea pig model of asthma

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Abstract

Since an allergen-induced early asthmatic reaction was found to be accompanied by oxidative stress and since levels of the endogenous antioxidant, glutathione, reportedly can be enhanced by a whey-supplemented diet (UWPC), it was investigated whether UWPC could alleviate allergen-induced lung contractions. To this end, guinea pigs were sensitized to ovalbumin (OVA) and fed water or UWPC in addition to standard lab chow. Blood samples were taken on several days after sensitization to measure serum levels of allergen-specific IgG_1 and IgG_2. After three weeks, lungs were isolated and perfused with buffer containing the allergen. Airway contractions were assessed, and several airway contractile and relaxant mediators, as well as indicators for oxidative stress, were measured in the lung effluent. Liver glutathione levels were determined to control for the GSH-enhancing effect of UWPC. UWPC feeding significantly enhanced liver glutathione by 20%, and significantly decreased lung contractions in sensitized animals by 45%. IgG levels were not altered by UWPC. The airway relaxant, PGE_2, was increased upon OVA-challenge in OVA-sensitized groups, and tended to be decreased by UWPC. The indicator of oxidative stress and airway contractile mediator, 8-*iso*-$PGF_{2\alpha}$, was also increased upon OVA challenge in OVA-sensitized groups, and tended to be further increased by UWPC. Another indicator of oxidative stress, thiobarbituric reactive substances (TBARS), was also increased in OVA-sensitized animals as compared to saline-sensitized controls. UWPC-feeding did not alter the levels of the OVA-sensitized group but tended to increase the TBARS levels of the saline-sensitized group. In conclusion, UWPC feeding reduced lung contractility, but this was probably not through an antioxidant effect of UWPC.

Introduction

Reactive oxygen species play a prominent role in inflammatory lung diseases, including asthma. Although the lungs are equipped with a variety of enzymatic and non-enzymatic anti-oxidant systems, there are indications that asthma is associated with a local oxidant / antioxidant imbalance, particularly during exacerbations of the disease. One of the most prominent endogenous antioxidants of the airways is glutathione (GSH). We have previously shown in a guinea pig model of allergic asthma that an allergen-induced early asthmatic response (EAR) is paralleled by markedly decreased lung GSH levels (chapter 4). In an isolated lung perfusion set up, we also demonstrated that the allergen-induced airway contractions could be prevented by a cell-permeable GSH analogue in the perfusing buffer (chapter 4).
To demonstrate the therapeutic relevance of these findings, it would be of interest to enhance glutathione levels of the airways *in vivo*. Administration of GSH, however, is not an effective way to increase tissue GSH levels (1), while the GSH prodrug, cysteine, is not useful because of its toxicity (2, 3). The cysteine derivative, N-acetylcysteine, is not toxic but its oral bioavailability is poor (see (4) for a review). An alternative way to enhance GSH levels *in vivo* is the food supplement *undenatured whey protein concentrate* (UWPC). UWPC is a mixture of proteins and peptides rich in γ-glutamylglycine, a dipeptide that is known to increase intracellular GSH (5). Interestingly, a recent case report has referred to a patient with obstructive lung diseases whose improvement of pulmonary function and increase in plasma GSH levels could be correlated to UWPC intake (6).

In the present study, the effect of UWPC was assessed using the above described ex vivo guinea pig model of allergic asthma. Guinea pigs were fed UWPC as a supplement from three days before sensitization with allergen until sacrifice, three weeks later. At that time, allergen-induced contractions of isolated lungs, as well as lung perfusate levels of indicators of oxidative stress and a number of contractile and relaxant arachidonic acid metabolites were measured. Furthermore, total glutathione content of the liver was measured. Blood samples were taken before and at regular intervals after sensitization to determine allergen-specific IgG levels in serum.

Methods

Animals
Male specific pathogen-free Dunkin Hartley guinea pigs (Harlan Nederland, Horst, The Netherlands), weighing 200 – 250 g at the start of the experiments were housed

under controlled conditions. Water and commercial chow were allowed *ad libitum*.

Ovalbumin sensitization and challenge

Animals were sensitized on day 0 by injecting a mixture of 20 μg ovalbumin (OVA; grade V, Sigma, St Louis, MO) and 200 mg of the adjuvant, $Al(OH)_3$ (Merck, Darmstadt, Germany), in 1.0 ml saline. $Al(OH)_3$ 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 given subcutaneously in the axillar and inguinal regions and in the nuchal area. On day 20, airways were isolated and challenged with OVA as described in the section *Tracheally perfused lung preparation*.

Diet regimen

UWPC was gently dissolved in sterile tap water at a concentration of 0.5 g/ml immediately before use. It was administered *per os* using a syringe with a polyethylene tubing that was placed at the back of the mouth of the animal. Slowly emptying the syringe made the animals swallow spontaneously. Each animal received 5 ml of whey solution twice daily, with 7 h between administrations. Controls received sterile tap water following the same administration regimen. Whey administration started three days before sensitization.

Tracheally perfused lung preparation

Tracheal perfusion was performed on day 20 after sensitization as previously described (7). 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 and the abdominal cavity was opened to inject 500 U of heparin into the vena cava and to severe and the abdominal aorta 3 min later. Then, the thoracic cavity was opened to remove heart and lungs *en bloc*. The lungs were dissected free and hung in a plexiglas box at 37ºC and 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, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.05 mM, KCl 2.68 mM, $NaHCO_3$ 0.6 mM, NaH_2PO_4 0.13 mM, and Na_2HPO_4 0.896 mM. The buffer was warmed to 45ºC and pumped at a rate of 2 ml/min through a bubble trap before being cooled to 37ºC 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 (7). 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 as described earlier (Chapter 4).

Measurement of liver glutathione
After lung isolation, a liver lobe was snap-frozen in liquid nitrogen from each animal to be stored at -80ºC until analysis. Each lobe was then crushed in liquid nitrogen using a mortar and pestle; the resulting powder was transferred to an eppendorf tube and a mixture of 1 M $HClO_4$ with 2 mM EDTA was added (1 ml per 250 mg powder). After vigorous vortex mixing, tubes were centrifuged at 5000 *g* for 10 min and total glutathione (GSH + GSSG) concentrations in the supernatants were determined with a modified version (8) of the glutathione reductase-DTNB recycling assay according to Akerboom *et al*. (9). Values were expressed in nmol per mg tissue (wet weight).

OVA-specific serum IgG
Blood samples (± 350 µl each) were collected from a femoral vein on days -2, 5, 12, 18, and 20. Blood was allowed to clot for 30 min at room temperature before being centrifuged at 20,000 *g* for 10 min. Serum was collected and stored at -20ºC until analysis. IgG_1 and IgG_2 antibody responses to OVA were assayed with an ELISA. Flat-bottom microplates (96-wells, Maxisorp, Nunc A/S, Roskilde, Denmark) were coated with 100 µg OVA per ml buffer (38 mM Na_2CO_3, 43 mM KH_2PO_4; pH 9.5) for 60 min at 37°C. After washing with 0.05% Tween20 in PBS, free binding sites were blocked with 1% BSA (Boehringer – Mannheim) in ELISA buffer (50 mM Tris, 2 mM EDTA, 136.9 mM NaCl, 0.05% Tween20; pH = 7.2) at 37°C for 60 min. After removal of the blocking buffer and washing, serially diluted serum samples were incubated for 2 h at 37°C. After washing, the plates were incubated with horseradish peroxidase-conjugated goat anti-guinea pig IgG_1 and IgG_2 (diluted 1/10,000 and 1/3,000 , respectively; Bethyl laboratories, Montgomery, TX) in ELISA buffer with 0.5% BSA. After washing, O-phenylene diamine dihydrochloride (0.4 mg/ml; Sigma) in PBS containing 0.012% hydrogen peroxide was added. The reaction was stopped after 10 min through addition of 4 M H_2SO_4. Optical density was measured at 490 nm with a Titrek Multiscan (Flowlabs, Irvine, UK). Serum samples that had been pre-incubated (120 min, 37°C) with UWPC (25 ng/ml) underwent the same procedure to control for cross-reactivity of OVA-specific IgG with UWPC, using preincubation with a similar amount of OVA as a positive control.

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 -80ºC until analysis. Within three weeks after collection, levels of PGE_2, $LTC_4/D_4/E_4$, and 8-*iso*-$PGF_{2\alpha}$were measured in the samples with commercial EIA kits (Cayman, Ann Arbor, MI for 8-*iso*-$PGF_{2\alpha}$; Amersham Pharmacia Biotech for the LT as well as for PGE_2 analysis) according to manufacturer's instructions. Samples (100 µl) for measurement of TBARS were acidified to pH 2 - 3 with 10 µl 1% (w/v) trichloroacetic acid. Protein was precipitated by centrifugation and 100 µl supernatant was allowed to react with an equal volume of thiobarbituric acid (0.67%, w/v) for 10 min at 100°C. After cooling to room temperature, the absorbance at 532 nm was measured photometrically (10). Perfusion buffer was treated the same way to serve as a blank.

Data analysis and statistics

Tracings reflected the increase in airway opening pressure as a function of time. For each animal, the area under the curve was calculated from 50 s to 150 s after addition of allergen to the buffer. If data were normally distributed, they were analyzed with a one-way ANOVA followed by the LSD post-hoc test for multiple comparisons. If data were not normally distributed, they were analyzed with the Kruskal-Wallis test. P-values < 0.05 were considered to reflect significant differences. The numbers of animals studied were 9 in both saline-sensitized groups, 7 in the water-fed OVA-sensitized group, and 8 in the UWPC-fed OVA-sensitized group.

Results

Liver glutathione

Livers from control-fed guinea pigs contained 2.3 nmol of total glutathione/mg wet tissue. UWPC-feeding induced a 20% increase of total glutathione content of the liver (figure 1; $P < 0.05$).

Airway contractions

In the lungs from non-sensitized groups, OVA perfusion did not change airway opening pressure from either control-fed or UWPC-fed animals. In contrast, OVA provoked clear airway contractions in lungs from sensitized animals (figure 2).

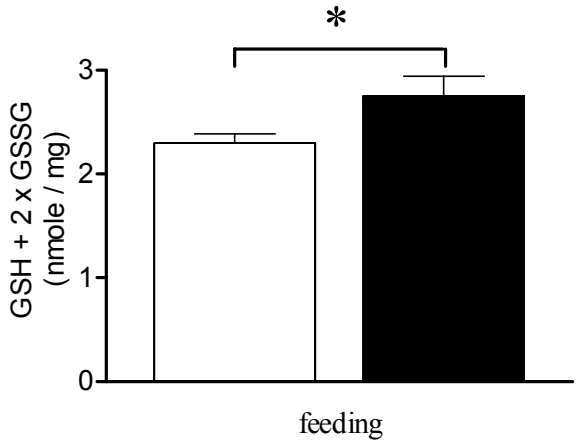

Figure 1. Levels of total glutathione (GSH + GSSG) in livers from water- (white bar) and UWPC- (black bar) fed guinea pigs. UWPC treatment enhanced liver levels of total glutathione as compared to livers of water-fed guinea pigs. N = 11 in the water-fed and 10 in the UWPC-fed group. Bars indicate mean ± SEM; * $P < 0.05$.

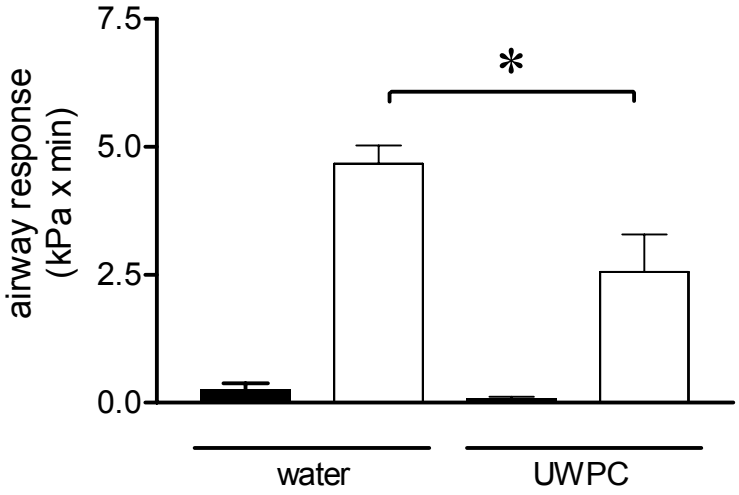

Figure 2. Lung contractility of isolated and perfused guinea pig lungs during the allergen-induced early asthmatic response. OVA challenge induced contractions in lungs from OVA-sensitized (white bars), but not saline-sensitized (black bars) guinea pigs, but the OVA-induced contractions were substantially reduced in the UWPC-fed group as compared to the water-fed group.
Bars indicate mean ± SEM; * $P < 0.05$.

UWPC-feeding reduced the allergen-induced airway contractions in sensitized animals by 45% as compared to sensitized animals that had received water ($P < 0.05$).

OVA-specific serum IgG

OVA-specific IgG antibodies could not be detected in sera from sham-sensitized animals (not shown). In sera of OVA-sensitized animals, a time-dependent increase in OVA-specific IgG_1 and IgG_2 was observed (figure 3). UWPC-feeding of sensitized animals did not alter their IgG plasma levels as compared to the sensitized water-fed group. Furthermore, pre-incubation of serum samples with UWPC did not affect binding of serum IgG to OVA-coated plates, while pre-incubation with OVA blocked the binding completely (data not shown).

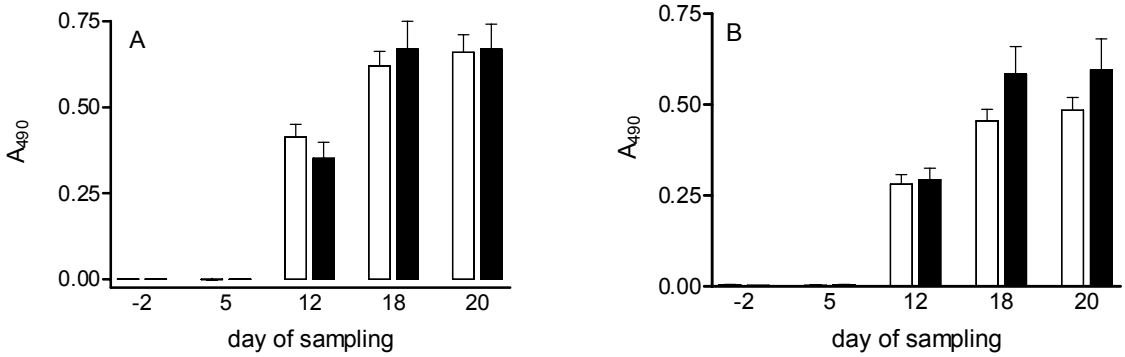

Figure 3. IgG_1 (panel A) and IgG_2 levels (panel B) in 1280-fold diluted serum of water-fed (white bars) and UWPC-fed (black bars) guinea pigs after sensitization to OVA. A time dependent, but feeding independent increase in IgG_1 and IgG_2 levels was observed.
Bars indicate mean ± SEM.

Effluent analysis

Allergen challenge of lungs from the water-fed sensitized animals increased PGE_2 in lung perfusates, as compared to non-sensitized animals ($P < 0.05$, figure 4). UWPC-treatment decreased PGE_2 levels in perfusates of sensitized guinea pig lungs by 55% as compared to the corresponding water-fed control group. This effect, however, did not reach statistical significance.

Histamine and leukotrienes were undetectable in almost all effluent samples both of saline- and OVA-sensitized groups.

Allergen challenge doubled 8-*iso*-$PGF_{2\alpha}$ levels in lung perfusate of water-fed sensitized animals, as compared to water-fed non-sensitized animals ($P < 0.05$, figure 5). UWPC-feeding did not significantly alter the 8-*iso*-$PGF_{2\alpha}$ levels, although they tended to be higher than those of the corresponding water-fed control group.

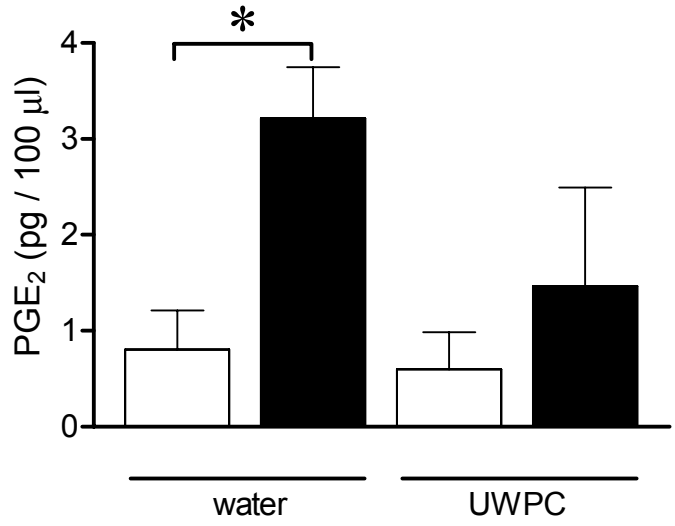

Figure 4. PGE_2 levels in perfusate samples taken during the allergen-induced early asthmatic response of isolated guinea pig lungs. OVA challenge increased PGE_2 formation in lungs from OVA-sensitized (black bars) animals as compared to saline-sensitized controls (white bars), but less so upon UWPC feeding.
Bars indicate mean ± SEM; * $P < 0.05$.

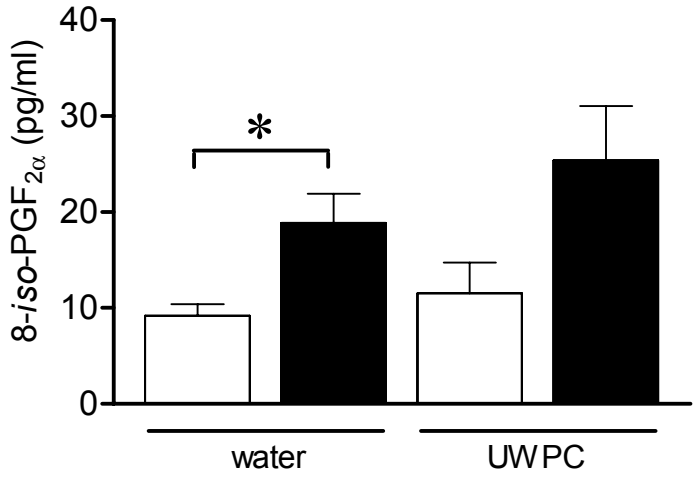

Figure 5. 8-*iso*-$PGF_{2\alpha}$ levels in perfusate samples taken during the allergen-induced early asthmatic response of isolated guinea pig lungs. OVA challenge increased 8-*iso*-$PGF_{2\alpha}$ formation in lungs from OVA-sensitized animals (black bars) as compared to saline-sensitized controls (white bars), and UWPC feeding tended to further increase these levels.
Bars indicate mean ± SEM; * $P < 0.05$.

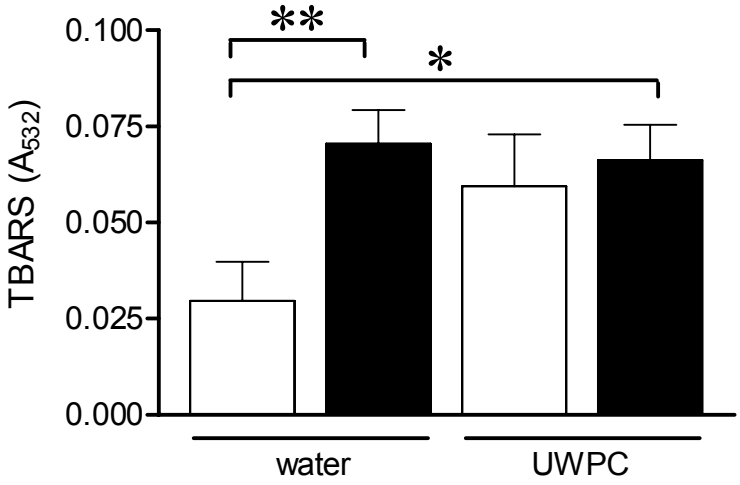

Figure 6. Levels of thiobarbituric acid reactive substances (TBARS) in perfusate samples taken during the allergen-induced early asthmatic response of isolated guinea pig lungs. OVA challenge increased TBARS formation in lungs from water-fed OVA-sensitized animals (left-hand black bar) as compared to water-fed saline sensitized controls (left-hand white bar). After OVA-challenge, TBARS levels in the UWPC-fed saline-sensitized group (right-hand white bar) and in the UWPC-fed OVA-sensitized group (right-hand black bar) were similar as those in the water-fed OVA-sensitized group. Bars indicate mean ± SEM; ** $P < 0.01$; * $P < 0.05$.

OVA challenge doubled absorbance due to TBARS in samples from OVA-sensitized water-fed group, as compared to the saline-sensitized group ($P < 0.01$, figure 6). UWPC feeding tended to increase TBARS levels of the saline-sensitized UWPC-fed group, and did not alter TBARS levels of the OVA-sensitized group, as compared to the corresponding water-fed group.

Discussion

This study has demonstrated that feeding a UWPC food supplement protects against early allergen-induced airway contractions in a guinea pig model of asthma (figure 2). Since serum levels of allergen-specific IgG_1 and IgG_2 were similar in UWPC-fed and water-fed animals, the reduced airway contractions could not be explained by an effect of UWPC on the production of these antibodies that reportedly mediate immediate type hypersensitivity in the guinea pig (11) (figure 3). Liver glutathione,

however, was increased by UWPC feeding (figure 1), suggesting increased GSH levels in other tissues, including the lungs, as well. If so, UWPC treatment may have decreased allergen-induced contractions via a similar mechanism as perfusion with GSH or a GSH-donor, which was earlier shown to relax airway smooth muscle (Chapter 3) and prevent allergen- and histamine-induced airway contractions (Chapter 4). Unfortunately, lung GSH levels could not be measured reliably, since the contractile state of the airway influenced the amount of buffer in the lungs, making it hard to relate GSH content to tissue weight.

The mechanism by which UWPC decreased the allergen-induced airway contractions remains elusive. The observation that an early asthmatic response is associated with decreased GSH levels and a disturbed GSH/GSSG balance in guinea pig lungs (Chapter 4) strongly suggested that allergen-induced airway contractions are accompanied by oxidative stress in the guinea pig. This suggestion is substantiated by observations that two indicators of oxidative stress, 8-*iso*-$PGF_{2\alpha}$ (12) (figure 5) and TBARS (13) (figure 6), were increased in the effluent of lungs during the early asthmatic response.

It is conceivable that allergen-induced oxidative stress modulates the production of relaxing or contractile mediators. In this study, OVA challenge of lungs from sensitized animals increased effluent levels of the airway relaxant, PGE_2, as compared to saline-sensitized controls. Feeding UWPC tended to reduce the PGE_2 production. This is consistent with the putatively increased antioxidant action of UWPC feeding, since PGE_2 production is known to be stimulated by oxidative stress (14) and inhibited by a ROS-scavenger (15). Alternatively, since post-OVA challenge PGE_2 levels were higher in OVA-sensitized than in saline-sensitized groups, the amount of PGE_2 production may relate to the degree of airway contraction. Indeed, it has been shown by others that allergen-induced contractions of guinea pig trachea were paralleled by histamine release, and were followed somewhat later by PGE_2 release (16). Thus, PGE_2 synthesis may be stimulated by airway contractions to counteract excessive airway constriction. Accordingly, the lesser contraction in the UWPC-fed groups would result in less PGE_2 synthesis (figure 4).

The contractile mediators, histamine and leukotrienes, which are known to be released upon allergen-induced mast cell activation (17) and whose release is potentiated by oxidative stress (18), appeared hardly or not at all detectable in the perfusate of OVA-challenged lungs from sensitized animals, regardless of the feeding. Possibly, the flow rate (2 ml/min) of the perfusion buffer caused too much dilution of mediators to detect them in the perfusate.

The isoprostane, 8-*iso*-$PGF_{2\alpha}$, however, could be measured unambiguously in the perfusate. Besides being an indicator of oxidative stress as already addressed above, 8-*iso*-$PGF_{2\alpha}$ is a potent airway contractant (19-21). Indeed, OVA challenge of sensitized lungs significantly increased 8-*iso*-$PGF_{2\alpha}$ release, suggesting that the OVA-induced contraction could have been mediated in part by this compound. UWPC feeding, however, did not reduce 8-*iso*-$PGF_{2\alpha}$ levels in the perfusate but, on the contrary, tended to cause a slight increase. These seemingly conflicting data may be explained by assuming that UWPC feeding decreases the contractile reactivity to 8-*iso*-$PGF_{2\alpha}$. Earlier findings that GSH can decrease tracheal hyperreactivity to another contractile agent, histamine (chapter 3), while GSH depletion can render the airways hyperreactive to this mediator (chapter 4), provide supportive evidence for this assumption.

Assuming that UWPC increases lung GSH levels, an explanation for the failure of UWPC to decrease 8-*iso*-$PGF_{2\alpha}$ levels is the dual action of GSH on 8-*iso*-$PGF_{2\alpha}$ synthesis. GSH as an antioxidant may inhibit all oxidative steps in the pathway of 8-*iso*-$PGF_{2\alpha}$ formation. The last step, however, is a reduction of a bicyclic endoperoxide (22). Consequently, the last step in the formation of 8-*iso*-$PGF_{2\alpha}$ can be promoted by GSH (23). Thus, this dual effect formation may explain the lack of effect of UWPC on the levels of 8-*iso*-$PGF_{2\alpha}$ and may have obscured the capacity of UWPC to diminish oxidative stress. To address this possibility, TBARS were measured in the same samples as used for 8-*iso*-$PGF_{2\alpha}$ measurement. Like 8-*iso*-$PGF_{2\alpha}$, TBARS are endoperoxide breakdown products, but their formation does not require GSH. TBARS levels, however, appeared increased in both OVA-sensitized groups and clearly tended to be increased in the saline-sensitized, UWPC-fed group (figure 6). Provided that TBARS are an adequate representation of oxidative stress (24), these data suggest that UWPC has no anti-oxidant action in the lungs and rather induces oxidative stress in this organ. Since saline-sensitized, UWPC-fed animals had no OVA-specific antibodies in their serum, the presumed oxidative stress in the lungs from these animals could not have resulted from immune complex formation after OVA challenge. Moreover, the presumed UWPC-induced oxidative stress appeared not additive to the oxidative stress induced by OVA-challenge of sensitized animals. Therefore, it is unclear how the oxidative stress was generated and how UWPC significantly diminished the early asthmatic reaction without diminishing oxidative stress in the lungs.

In conclusion, although UWPC feeding reduced lung contractility, there are no data to substantiate that this was through an antioxidant effect of UWPC.

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6

Modulation of hyperresponsiveness by glutathione in a murine *in vivo* model of allergic asthma

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Abstract

It is becoming increasingly clear that oxidative stress contributes to the pathogenesis of asthma. Therefore, this study addressed the question whether supplements of the endogenous antioxidant, glutathione (GSH), in a murine allergic asthma model would alleviate features of asthma. To this end, sensitized mice received aerosols of the GSH-donors, glutathione-ethyl ester (GSEt) or N-acetylcysteine, before or during respiratory allergen challenges, or during cholinergically provoked airway contractions one day after the last allergen challenge. Lung GSH levels were measured shortly after allergen challenge or after cholinergic provocation, and broncho-alveolar lavages (BAL) were performed to study the effect of GSH supplements on the influx of inflammatory cells into the airways. GSEt was found to decrease allergen-induced cholinergic airway hyperresponsiveness when given in combination with methacholine. However, when given before or during allergen challenge, both GSH-donors failed to decrease subsequent methacholine-induced airway contractility, BAL cell numbers, or increase lung GSH levels. In addition, allergen challenges of sensitized mice did not decrease lung GSH levels, suggesting that these challenges do not cause oxidative stress. Therefore, it is concluded that the failure of GSH-donors to decrease allergen-induced cholinergic hyperreactivity when given before or during allergen challenge may be explained by the lack of oxidative stress during the allergen challenge. In view of the oxidative stress upon allergen challenge observed earlier in the guinea pig and the importance of oxidative stress in the pathology of allergic asthma in humans, the guinea pig may be a more suitable species than the mouse to study oxidative stress in asthma.

Introduction

Asthma is characterized by a chronic inflammatory condition of the lungs and reversible airflow obstruction. The inflammation is mild during remissions, but during exacerbations, inflammatory cells can produce large amounts of mediators, including reactive oxygen species (ROS). Exaggerated production of ROS may deplete anti-oxidant systems, leading to tissue damage and a disturbed biochemistry. This condition is referred to as oxidative stress and is increasingly appreciated in asthma (1, 2). It is still unclear how oxidative stress in asthma affects lung levels of glutathione (GSH), a prominent endogenous anti-oxidant. We recently demonstrated in a guinea pig model of asthma that allergen challenge induced GSH depletion of lung tissue during the early asthmatic response. In addition, supplementing GSH during an early asthmatic response in a lung perfusion model could prevent acute allergen-induced contractions. Besides allergen-induced acute airway contractions, airflow obstruction in asthmatics is manifest by exaggerated responses to contractile stimuli. This airway hyperreactivity (AHR) has a chronic character. Like allergen-induced acute airway contractions, AHR is generally considered to be a consequence of the inflammatory state of the lungs (3). Although the events contributing to its development are still ill understood, previous experiments in our lab have shown that isolated perfused lungs from GSH-depleted guinea pigs were hyperresponsive to histamine. In addition, perfusion of the lungs with GSH-ethyl ester (GSEt) prevented this hyperresponsiveness. Since these data suggest that GSH depletion may underlie AHR, the current study was done to address the question to what extent nebulization of GSH or GSH-precursors could counteract the development of airway inflammation and AHR in an *in vivo* murine model of asthma.

Methods

Animals

Animal care and use was in accordance with the guidelines of the local ethical committee for animal experiments. Specific pathogen-free male BALB/c mice (6 weeks) were obtained from the Utrecht University animal facilities department (Utrecht, The Netherlands) or from Charles River (Someren, Netherlands; study of protocol IV). The animals were housed in macrolon cages in a laminar flow cabinet and provided with food and water ad libitum.

Ovalbumin sensitization and challenge

All animals were sensitized with an intraperitoneal injection (100 µl) of a mixture of 20 µg ovalbumin (grade V, Sigma, St Louis, MO; OVA) and 200 mg of the adjuvant, $Al(OH)_3$, in 1 ml saline on days 0 and 7. On days 30, 33 and 36, the animals were given a challenge through inhalation of a nebulized OVA solution (1%, w/v) for 30 min. Control animals received saline instead of OVA. The aerosol (particle size 2.5 - 3.1 µm) was generated in a plexiglas exposure chamber with a Pari LC star nebulizer (Pari Respiratory equipment, Richmond, VA, USA) that was driven by compressed air (6 l/min).

Intervention with thiol compounds

Thiol compounds GSEt or N-acetyl cysteine (NAC) (Sigma, St. Louis, MO, USA) were nebulized in the above-mentioned nebulizer. Thiols were dissolved at 100 mM in saline; each solution was adjusted to pH 7. Controls received vehicle.

Four treatment protocols were tested:

I. GSEt aerosol for 5 min, 6 h before each OVA or saline challenge.
II. GSEt aerosol for 5 min, 10 min before each OVA or saline challenge.
III. Aerosol of a mixture of NAC and OVA for 30 min during each challenge.
IV. GSEt together with methacholine during provocation of airway contractions, 24 hours after the last OVA aerosol.

In protocols I and II, each group consisted of three animals, while in protocols II and IV, eight animals per group were used.

Measurement of airway responsiveness

Twenty-four hours after the last allergen challenge, airway responsiveness was measured in conscious, unrestrained mice using barometric whole-body plethysmography (Buxco, EMKA Technologies, France). Enhanced pause (Penh) was taken as a measure of airway responsiveness, as described in detail previously (4). Mice were exposed to increasing concentrations of nebulized methacholine (acetyl-β-methylcholine chloride, Sigma, St. Louis, MO, USA; 1.6 - 13 mg/ml saline) in a plexiglas Buxco chamber. Each dose of methacholine was nebulized for 3 min and followed by a 3-min-period during which airway responses were recorded.

Broncho-alveolar lavage

Following airway responsiveness measurements, mice received a lethal dose of pentobarbitone sodium (Euthesate® 0.6 g/kg body weight, intraperitoneally). The

trachea was trimmed free of connective tissue, and a small incision was made to insert a cannula in the trachea. Via this cannula, the lavage was performed by filling the lungs with 1.0 ml sterile saline of 32°C. and withdrawing it after 10 seconds. The cell suspension was collected in a plastic tube on ice. This procedure was repeated three times to obtain a pooled cell suspensions from each animal. The cells were spun down by centrifugation at 400 *g* for 5 minutes at 4°C and resuspended in 150 µl ice-cold PBS. A sample of this was stained with Türk's solution and counted in a Bürker-Türk bright-line counting chamber to calculate the total number of cells. Another sample was used to prepare cytospin preparations. These were air dried, fixed, and stained with Diff-Quik (Merz + Dade A.G., Düdingen, Switzerland). The cells were differentiated morphologically into alveolar macrophages, eosinophils, lymphocytes and monocytes, and neutrophils by light microscopical observation.

Glutathione measurements

After the broncho-alveolar lavage, lungs were isolated and snap frozen in liquid nitrogen until analysis for GSH and oxidized glutathione (GSSG). To that end, lungs were crushed in liquid nitrogen using a mortar and pestle; the resulting powder was divided between two eppendorf tubes. To one tube, a mixture of 1 M $HClO_4$ 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 (NEM) was added to the other tube to assess levels of oxidized glutathione. After vigorous vortex mixing, the tubes were centrifuged at 5000 *g* for 10 min and total and oxidized glutathione concentrations in the supernatants were determined with a modified version (5) of the glutathione reductase-DTNB recycling assay according to Akerboom *et al*. (6). Values were expressed in nmol per g tissue (wet weight).

Influence of OVA challenge on lung GSH/GSSG levels

To investigate the effect of OVA challenge on lung GSH and GSSG levels, animals were sacrificed with pentobarbitone sodium (Euthesate® 0.6 g/kg body weight, intraperitoneally) immediately after the first or the third OVA aerosol, or 24 hours after the third challenge. Lungs were quickly isolated and snap-frozen in liquid nitrogen to be assayed for GSH and GSSG levels. Eight animals were used per time point.

Statistics

Dose response curves were statistically analyzed using a general linear model of repeated measures test, followed by the LSD post-hoc test for multiple comparisons.

In case the GSH levels and BAL cell counts were normally distributed, they were analyzed with a one-way ANOVA followed by the LSD post-hoc test for multiple comparisons. In case data were not normally distributed, they were analyzed with the Kruskal-Wallis test. Differences were considered statistically significant if $P < 0.05$.

Results

In animals that were treated with vehicle instead of GSEt 6 h before each challenge, saline challenge resulted in a normal dose-dependent increase of airway reactivity to methacholine (figure 1A and B).

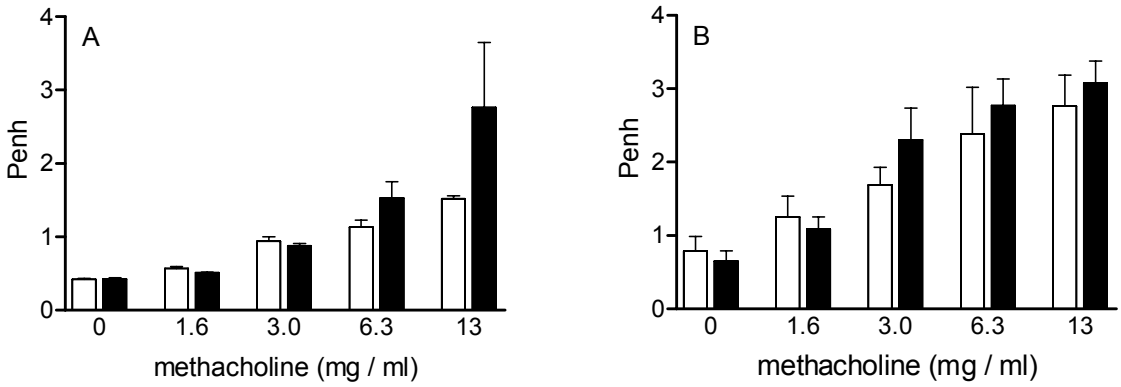

Figure 1. Methacholine-induced airway contractions in OVA-sensitized mice that received vehicle (white bars) or GSEt (black bars) 6 h before each challenge with saline (panel A) or OVA (panel B). OVA challenges induced significant ($P < 0.05$) hyperresponsiveness as compared to saline challenges. GSEt-treatment had no influence on airway contractions of saline-challenged mice; neither did it alter OVA-induced hyperresponiveness.
Bars indicate mean ± SEM

Compared to saline challenge, OVA challenge caused cholinergic hyper-responsiveness ($P < 0.05$). GSEt treatment did not affect cholinergic responsiveness as compared to the corresponding vehicle treatment.
Lungs from the vehicle-pretreated, saline-challenged mice contained 300 and 90 nmol/g wet tissue weight GSH and GSSG, respectively (figure 2). These levels were not significantly changed by GSEt pretreatment or OVA challenge.

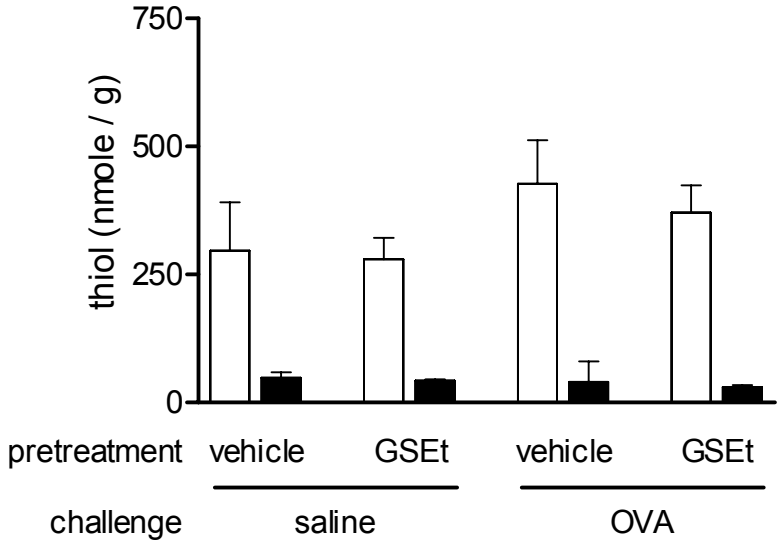

Figure 2. GSH (white bars) and GSSG (black bars) levels of lung tissue from OVA-sensitized mice that received vehicle or GSEt 6 h before each challenge with saline or OVA. GSEt-treatment did not increase lung GSH of both saline- and OVA-challenged mice. Bars indicate mean ± SEM.

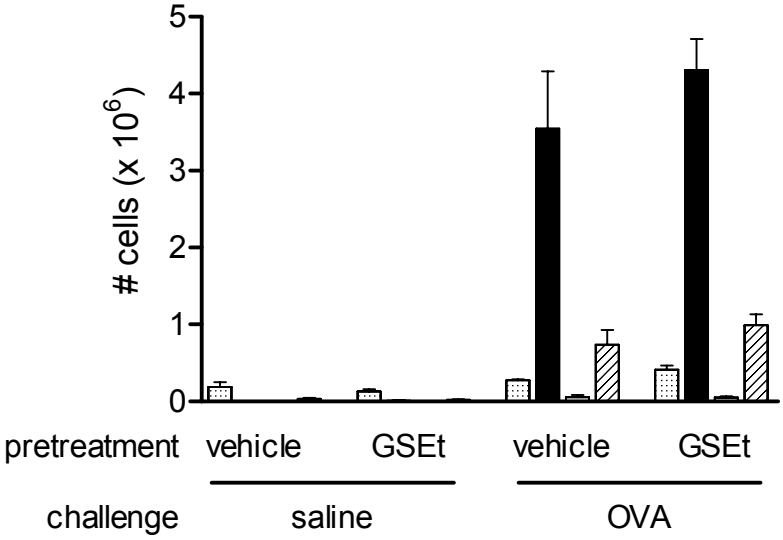

Figure 3. Numbers of inflammatory cells in broncho-alveolar lavage fluid from OVA-sensitized mice that received vehicle or GSEt 6 h before each challenge with saline or OVA. OVA challenge significantly ($P < 0.01$) increased the number of inflammatory cells. Neither basal cell counts nor the OVA-induced increase of cell numbers were affected by GSEt-treatment. Dotted bars, macrophages; black bars, eosinophils; white bars, neutrophils; hatched bars, lymphocytes and monocytes.
Bars indicate mean ± SEM.

Lung lavage fluid from the vehicle-pretreated, saline-challenged mice contained normal leukocyte numbers, largely consisting of macrophages (figure 3). OVA challenge significantly ($P < 0.01$) increased total cell numbers as particularly caused by an increased number of eosinophils and, to a lesser extent, lymphocytes and monocytes. GSEt pretreatment did not significantly change total and differential cell numbers as compared to the vehicle pretreatment.

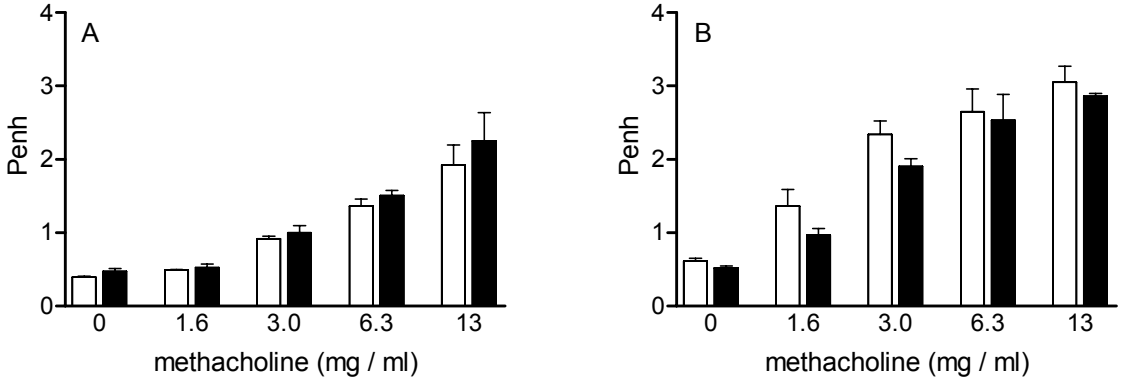

Figure 4. Methacholine-induced airway contractions in OVA-sensitized mice that received vehicle (white bars) or GSEt (black bars) 5 min before each challenge with saline (panel A) or OVA (panel B). OVA challenges induced significant ($P < 0.01$) hyperresponsiveness as compared to saline challenges. GSEt-treatment did not alter basal airway responses in saline challenged animals or OVA-induced hyperresponiveness. Bars indicate mean ± SEM.

In animals that were treated with vehicle instead of GSEt 10 min before each challenge, saline challenge resulted in normal airway reactivity to methacholine (figure 4A and B) while OVA challenge caused cholinergic hyperresponsiveness ($P < 0.01$). GSEt treatment did not affect cholinergic responsiveness as compared to the corresponding vehicle treatment.

GSH and GSSG levels of lungs from the vehicle-pretreated, saline-challenged mice were 390 and 16 nmol/g, respectively (figure 5), and were not significantly changed by GSEt pretreatment or OVA challenge.

Lung lavage fluid from the vehicle-pretreated, saline-challenged mice contained normal leukocyte numbers, mainly macrophages (figure 6). OVA challenge significantly ($P < 0.01$) increased total cell numbers, eosinophils and lymphocytes and monocytes in particular. GSEt pretreatment did not significantly change total and differential cell numbers as compared to the vehicle-pretreated groups.

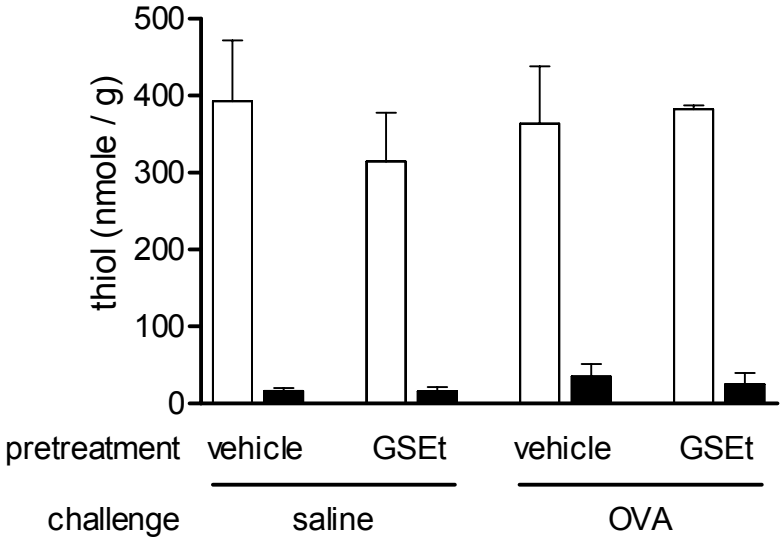

Figure 5. GSH (white bars) and GSSG (black bars) levels of lung tissue from OVA-sensitized mice that received vehicle or GSEt 5 min before each challenge with saline or OVA. GSEt-treatment did not increase lung GSH of either saline- or OVA-challenged mice. Bars indicate mean ± SEM.

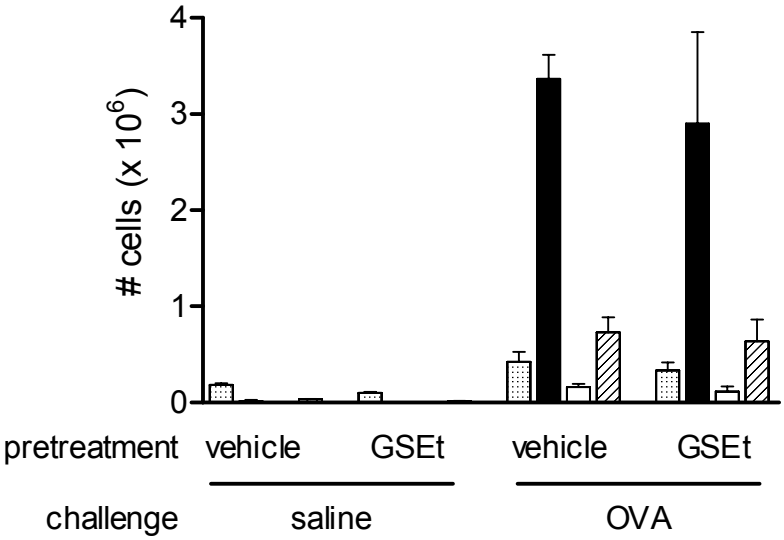

Figure 6. Numbers of inflammatory cells in broncho-alveolar lavage fluid from OVA-sensitized mice that received vehicle or GSEt 5 min before each challenge with saline or OVA. OVA challenge significantly ($P < 0.05$) increased the number of inflammatory cells; this increase was not moderated by GSEt-treatment. Dotted bars, macrophages; black bars, eosinophils; white bars, neutrophils; hatched bars, lymphocytes and monocytes. Bars indicate mean ± SEM.

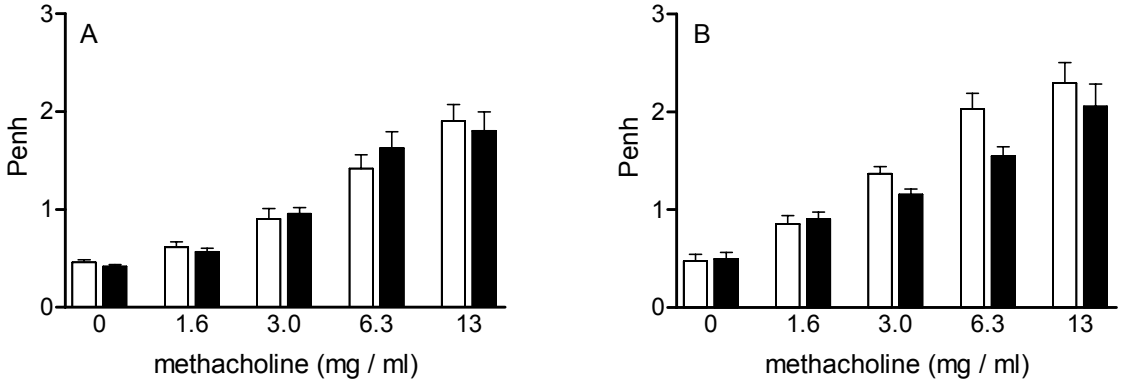

Figure 7. Methacholine-induced airway contractions in OVA-sensitized mice that received vehicle (white bars) or NAC (black bars) mixed with each saline (panel A) or OVA (panel B) challenge. OVA challenges induced significant ($P < 0.01$) hyperresponsiveness as compared to saline challenges. NAC did not alter airway contractions of saline-challenged mice, or OVA-induced hyperresponiveness significantly. Bars indicate mean ± SEM.

In animals challenged with saline aerosol without NAC, airway reactivity to methacholine was normal (figure 7A), while cholinergic hyperresponsiveness was observed after challenge with OVA aerosol (figure 7B; $P < 0.05$). Addition of NAC to the saline aerosol did not affect the airway response to methacholine. However, the addition of NAC to the OVA aerosol tended to decrease the hyperresponsiveness, but the effect was not statistically significant.
Lungs from the saline-challenged mice contained 580 and 47 nmol/g GSH and GSSG, respectively (figure 8). These levels were not significantly changed by NAC or OVA challenge.

Airway responses to methacholine and to methacholine mixed with GSEt did not differ in animals challenged with saline instead of OVA (figure 9A). In OVA-challenged mice, however, the hyperresponsiveness to methacholine was significantly attenuated by the addition of GSEt to the methacholine ($P < 0.05$).
Lungs from mice challenged with methacholine one day after saline challenge contained 270 and 23 nmol/g GSH and GSSG, respectively (figure 10). These levels were not significantly changed by the addition of GSEt to the methacholine, or by the OVA challenge.
OVA challenge significantly ($P < 0.01$) increased lung leukocyte numbers, particularly eosinophils and lymphocytes and monocytes, as compared to saline challenge. The cell numbers were not changed by challenge with methacholine or methacholine together with GSEt (figure 11).

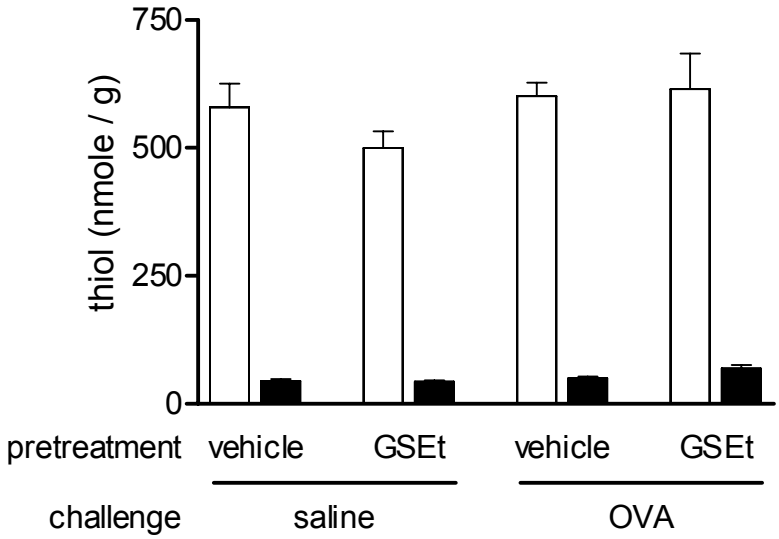

Figure 8. GSH (white bars) and GSSG (black bars) levels of lung tissue from OVA-sensitized mice that received vehicle or GSEt mixed with each saline or OVA challenge. NAC did not increase lung GSH of either saline- or OVA-challenged mice. Bars indicate mean ± SEM.

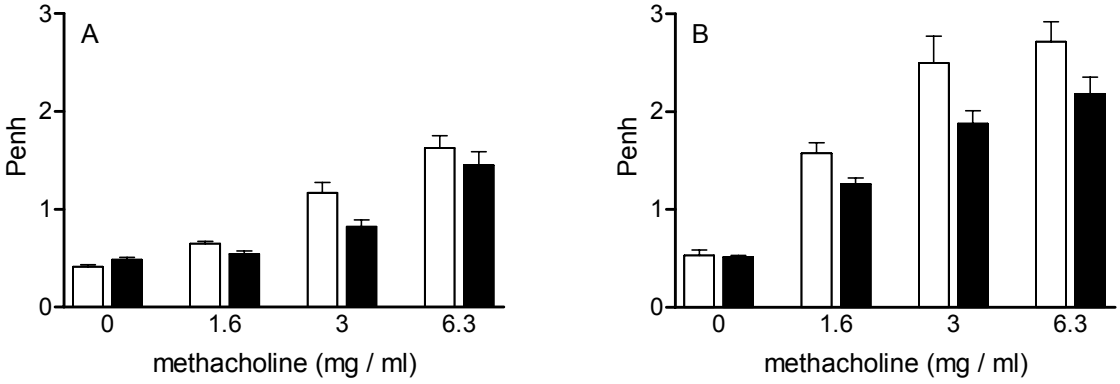

Figure 9. Methacholine-induced airway contractions in OVA-sensitized mice that received vehicle (white bars) or GSEt (black bars) mixed with methacholine, 24 h after the last challenge with saline (panel A) or OVA (panel B). OVA challenges induced significant ($P < 0.01$) hyperresponsiveness as compared to saline challenges. GSEt did not alter normal airway responses in saline-challenged mice, but it significantly ($P < 0.05$) decreased the OVA-induced hyperresponsiveness to methacholine.
Bars indicate mean ± SEM.

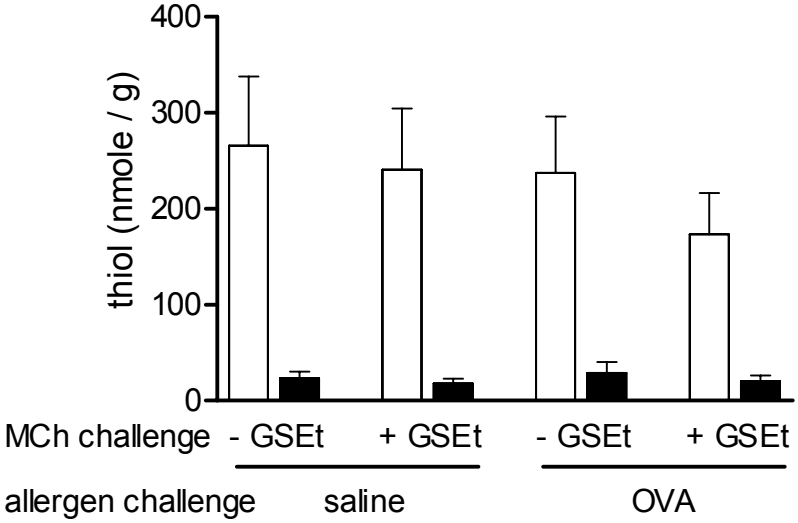

Figure 10. GSH (white bars) and GSSG (black bars) levels of lung tissue from OVA-sensitized mice that received vehicle or GSEt mixed with methacholine, 24 h after the last challenge with saline or OVA. GSEt-treatment did not increase lung GSH of either saline- or OVA-challenged mice. Bars indicate mean ± SEM.

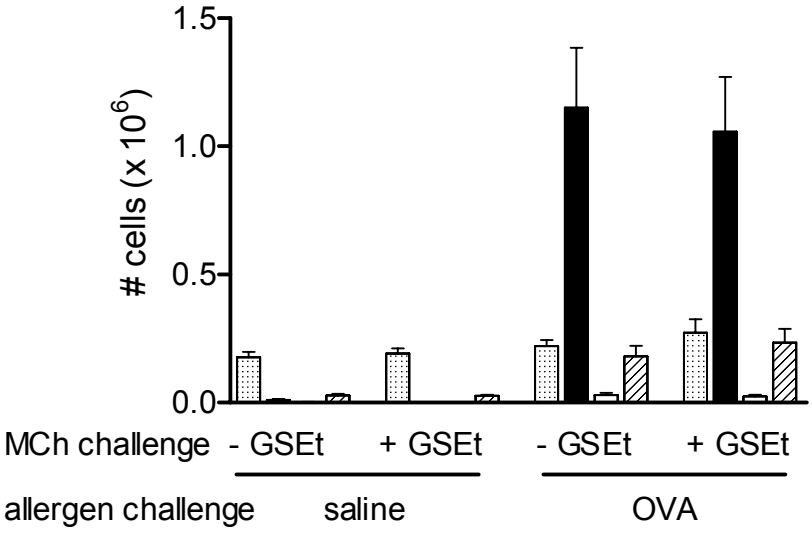

Figure 11. Numbers of inflammatory cells in broncho-alveolar lavage fluid from OVA-sensitized mice that received vehicle or GSEt mixed with methacholine, 24 h after the last challenge with saline or OVA. OVA challenge significantly ($P < 0.01$) increased the number of inflammatory cells; this increase was not moderated by GSEt treatment. Dotted bars, macrophages; black bars, eosinophils; white bars, neutrophils; hatched bars, lymphocytes and monocytes. Bars indicate mean ± SEM.

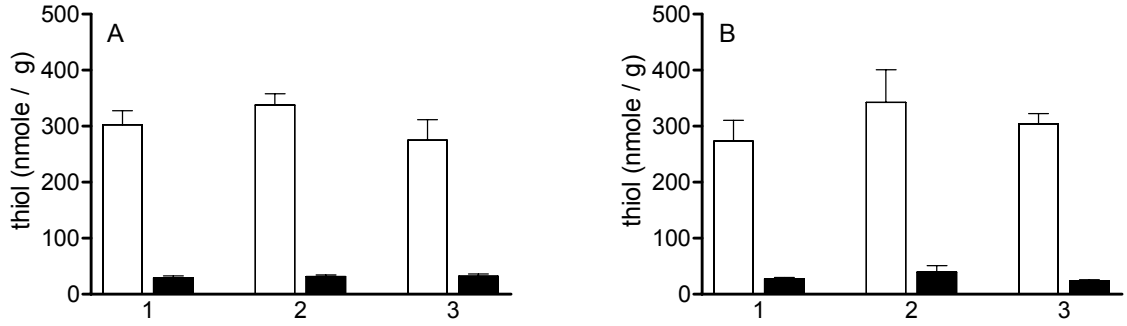

Figure 12. Influence of challenges with saline (panel A) or OVA (panel B) on tissue levels of GSH (white bars) and GSSG (black bars) in lungs of OVA-sensitized mice at different time points: 1, immediately after first challenge; 2, immediately after last challenge; 3, twenty-four hours after the last challenge. Neither GSH levels nor GSSG levels were significantly altered at any time point. Bars indicate mean ± SEM.

Influence of OVA challenge on lung GSH/GSSG levels

GSH and GSSG levels were not significantly changed immediately after the first or third OVA challenge, or 24 h after the latter challenge, as compared to challenge with saline (figure 12A and B).

Discussion

This chapter describes the effects of the GSH-donors, GSEt and NAC, in a murine model of allergic asthma on airway responsiveness, lung GSH and GSSG levels, and on the influx of inflammatory cells in the airways.

Administration of the GSH-donors before or during allergen-challenge appeared to be an inadequate way to moderate airway hyperresponsiveness, although NAC given during OVA challenge tended to decrease airway hyperresponsiveness (figure 7). In these studies, the GSH-donors did not increase lung GSH and GSSG levels as measured one day after the last OVA challenge, immediately after the assessment of cholinergic airway responses (figures 2, 5, and 8). The unchanged GSH levels and BAL values one day after the last allergen challenge, and the observation that addition of GSEt to methacholine decreased cholinergic responsiveness (figure 9) probably explains the lack of effect of GSH donors on airway contractility when given before or during allergen challenge. The design of these treatment protocols was based on previous experiments in guinea pigs, showing a clear drop in lung GSH levels and an increase in GSSG levels during an OVA-induced early asthmatic

response. In OVA-sensitized mice, however, allergen challenge did not affect lung GSH levels (figure 12), suggesting that there is no need to replenish GSH during allergen challenge in mice. The combined absence of GSH-depletion and a symptomatic early asthmatic response upon allergen challenge in mice versus the presence of both features in guinea pigs (chapter 4), apparently reflects a species difference. This observation also supports the conclusion in chapter 4 that an early asthmatic response and a decrease of lung GSH are related.

Although GSH supplementation decreased cholinergic responsiveness only when given during methacholine challenge, the protocols in this paper provide useful information regarding future studies on administration of GSH donors. The failure of GSH donors to enhance GSH levels in the lung can be explained in several ways. Firstly, the assessment of lung GSH levels, 24 h after the last treatment, was possibly too late to detect a, possibly transient, increase. Additionally, GSH supplementation may have led to a compensatory feedback inhibition of GSH synthesis, since GSH is a feedback inhibitor of its own synthesis (7). Secondly, the effect of GSEt nebulization may have been limited to the epithelium and the sub-epithelial tissues. Since GSH levels have been measured in whole lungs, an increase of GSH in (sub-) epithelial tissues may have been obscured. Finally, the GSH-donors may not have entered the tissue, but instead may have remained in the epithelial lining fluid. In the protocol in which GSEt was administered in combination with methacholine challenges, the broncho-alveolar lavage had probably washed away most of the GSEt.

OVA challenges consistently increased BAL cell counts in the sensitized mice but, although such an inflammatory cell influx has been related to oxidative stress (8, 9), anti-oxidant treatment around the time of each OVA challenge did not change BAL cell numbers in our experiments. This may also be related to an absence of oxidative stress during the allergen challenge as indicated by the lack of GSH-depletion in the lungs (figure 12). In view of the evidence for an oxidative imbalance in a guinea pig model of allergic asthma (chapters 4 and 5), as well as in asthmatics (2, 10-13), the role of oxidative stress in the recruitment of inflammatory cells to the airways may be smaller in mice as compared to guinea pigs and humans.

It is concluded that the decrease in airway contractility in mice when GSEt was given in combination with a contractile agonist is in line with earlier experiments in guinea pigs. However, the murine model lacks an early asthmatic response and concomitant GSH depletion in the lungs, while these features are both prominent in guinea pigs. In view of the presence acute asthmatic responses in humans and the

increasing evidence for a role of oxidative stress in asthmatics, guinea pig models may be more suitable than murine models to study the relation between oxidative stress and allergic asthma.

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7

Summarizing discussion

This thesis has demonstrated a role for GSH in relaxing airway smooth muscle, as well as in moderating airway contractions. Although only a small number of researchers have looked at GSH from a perspective of regulation of smooth muscle tension, some data suggest a role for GSH in this area. Cheung *et al.* demonstrated in 1997 that GSH could dose-dependently relax coronary arteries (1). Conversely, GSH deficiency is associated with impaired relaxation. For instance, a high-cholesterol diet was reported to decrease vascular GSH levels in rabbit aorta in parallel with decreased responses to relaxing agents (2). Moreover, similar effects were produced by an inhibitor of GSH reductase or a thiol-oxidizing agent, and relaxations could be partially restored by GSEt (2). In another study, GSH-depletion by inhibition of its synthesis led to hypertension in rats (3). This effect could be mitigated by concomitant administration of the vitamins C and E. In line with these reports, BSO-induced GSH depletion has produced arterial narrowing in rats (4). While these papers point to a role of GSH in maintenance of vascular tone, the evidence for similar effects in the airways is less abundant and more indirect. The oxidizing agent, *t*-butylhydroperoxide, caused dose-dependent contractions in human bronchial muscle *in vitro*, which could be prevented by NAC, an antioxidant and GSH-precursor (5). A role of GSH as a protector against bronchoconstriction in asthmatics is suggested by a study that demonstrated an increasing odds ratio for asthma with increasing use of acetaminophen (6), an agent that depletes GSH in lungs of animals (7).

The GSH-induced decrease in tracheal tension is mediated by potassium channels

The mechanism(s) via which GSH relaxes smooth muscle or moderates its contractions are still elusive. Several mechanisms have been addressed in the studies mentioned earlier. In the study by Cheung *et al.* (1), GSH-induced relaxations of coronary arteries could be attributed to the smooth muscle relaxant, nitric oxide (NO), and its biochemical target, soluble guanylyl cyclase (sGC).

Indeed, GSH and other thiols can bind to NO (8, 9), thereby enhancing the half-life and bioavailability of the latter compound (10). Thus, after observing GSH-induced relaxations in perfused isolated trachea tubes of guinea pigs (chapter 3), an attempt was made to prove the involvement of NO. Increased nitrite concentrations in the efferent perfusate suggested mediation by NO, since nitrite is a stable NO breakdown product (11). However, despite the GSH-induced increased nitrite levels in the trachea effluent, scavenging nitric oxide or using an inhibitor of sGC did not decrease GSH-induced relaxations; this suggests that GSH-induced relaxation of smooth muscle cells in the trachea is caused by a different mechanism as compared to vascular tissue. In addition, the findings in chapter 3 indicate that relaxations that

are paralleled by increased nitrite levels are not necessarily NO-mediated. Indeed, the increased nitrite levels described in chapter 3 appeared to be an epiphenomenon. This phenomenon could be accounted for by a pathway presented by Singh *et al.* (12), in which the reaction between GSH and GSNO yields nitrite without an involvement of NO, in the presence of oxygen. Since the oxygen tension was high in the Krebs perfusion buffer, this pathway is likely to have occurred in the experiments described in chapter 3. The presence of nitrosylated cysteine residues in proteins and smaller thiols in the tracheal wall is very likely, because the tracheas were isolated from healthy guinea pigs and because the tracheal epithelium, which is an important source of NO (13), was removed shortly before the experiments. GSH would then have liberated NO from these stores in a reaction that did not permit NO to decrease trachea tension.

The involvement of potassium (K^+-)channels in the relaxation of smooth muscle by GSH (chapter 3) is supported by some comparable studies in which other antioxidants were used. For instance, the antioxidant, ascorbic acid, was shown to induce vasodilation in human veins *in vivo*. This effect could be blocked by the non-selective K^+-channel blocker, quinidine, but not by the specific blocker of ATP-dependent K^+-channels, glibenclamide (14). Likewise, a mimetic of the antioxidant systems, superoxide dismutase and catalase, was reported to protect the acetylcholine-induced relaxation of rat aortic rings from inhibition by superoxide. This protective effect was inhibited by glibenclamide or the non-selective K^+-channel blocker, TEA (15). Furthermore, reducing agents, including GSH, increase the open probability of Ca^{2+}-activated K^+-channels (16, 17), be it in clamped inside-out membrane patches, and with administration of GSH at the cytosolic side. In the experiments described in chapter 3, extracellular GSH may have increased cytosolic GSH via an active uptake mechanism. There is indeed some evidence for the presence of γ-glutamyl transpeptidase in the membrane of guinea pig trachea smooth muscle cells (18), the enzyme that mediates GSH transport into the cell. However, the almost immediate decrease of tracheal tension upon addition of GSH to the organ baths would implicate a very high turnover rate.
The identification of the exact subtypes of K^+-channels involved in the experiments described in chapter 3 requires further experiments with selective K^+-channel inhibitors.

Allergen-induced oxidative stress in the lungs
It was unclear whether the studies on perfused tracheas were indicative of actions of GSH in isolated lungs or *in vivo*. Notably, studies described in chapter 4 clearly demonstrated that an EAR in guinea pigs *in vivo* is paralleled by oxidative stress as

indicated by decreased lung GSH levels, increased GSSG levels and hence a disturbed balance between reduced and oxidized glutathione. These data strongly suggest that oxidative stress is a feature of an EAR. More evidence for that has been provided in chapter 5, where it was demonstrated that the indicators of oxidative stress, 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*$PGF_{2\alpha}$) and thiobarbituric acid reactive substances (TBARS), were increased in perfusate of isolated lungs during an EAR.

The source of oxidative stress is still elusive. Infiltrated leukocytes are the primary source of reactive oxygen species during an inflammatory reaction (19), and are thought to be responsible for the oxidative stress in the lungs of asthmatics (20, 21). However, during the very first EAR, which was investigated in the models of allergic asthma in chapters 4 and 5, leukocytes had not yet been recruited to the lungs. Possible other candidates for the generation of oxidative stress are the resident leukocytes, mast cells and macrophages. Indeed, it has been shown that histamine release by isolated and stimulated mast cells is accompanied by an increase in TBARS (22). Furthermore, activated macrophages are a well-established source of ROS in the lungs (23) and in an EAR, mast cell-derived TNF-α may activate macrophages to produce ROS (24, 25).

Regardless of its source, the oxidative stress can modulate airway contractility in at least two ways. It can either alter the formation of relaxing or contractile mediators, or modulate the responsiveness of smooth muscle to these mediators.

Modulation of the formation of contractile mediators by oxidative stress
Regarding oxidant effects on the formation of contractile mediators, histamine release from cultured rat mast cells reportedly requires oxidation of arachidonic acid by oxygen-centered free radicals (26). In addition, hydrogen peroxide has induced histamine release by isolated mast cells (27). An important group of contractile mediators are leukotrienes (28), which are generated from arachidonic acid by lipoxygenases. Interestingly, a number of classes of lipoxygenase inhibitors are antioxidative agents that act via reduction of the non-heme iron of the enzyme (29-32). Another arachidonic acid-derived product, largely formed non-enzymatically, is 8-*iso*-$PGF_{2\alpha}$. Its formation requires reactive oxygen species; accordingly, this compound was mentioned earlier in this chapter as an indicator of oxidative stress. However, it is also a potent airway contractant of animal and human airways (33-35).
The fact that the production of the above-mentioned contractile mediators is associated with oxidative stress suggests that their formation can be inhibited by antioxidants, possibly including GSH. In the case of cysteinyl-leukotrienes,

however, it should be noted that GSH itself is a substrate for the formation of these compounds; therefore, supplementing GSH may actually promote, rather than inhibit, leukotriene synthesis. Since leukotrienes could not be measured reliably (chapter 5), no final conclusions regarding the influence of putatively UWPC-increased GSH levels on leukotriene formation could be drawn. Similarly, an inhibitory effect of GSH on histamine levels in the lung could not be substantiated. Possibly, a lower flow rate of the perfusion buffer would increase the chance that mediators can be detected reliably. In contrast to leukotrienes and histamine, 8-*iso*-$PGF_{2\alpha}$ could be detected unambiguously (chapter 5). Although increased levels of 8-*iso*-$PGF_{2\alpha}$ confirmed the presence of oxidative stress during an EAR, this increase was not diminished by feeding UWPC that enhanced liver GSH levels. As discussed in that chapter, the failure of UWPC to decrease 8-*iso*-$PGF_{2\alpha}$ levels may be due to the dual action of GSH on 8-*iso*-$PGF_{2\alpha}$ synthesis. GSH as an antioxidant may inhibit all oxidative steps in the pathway of 8-*iso*-$PGF_{2\alpha}$ formation, except the last one. Since the last step involves the reduction of a bicyclic endoperoxide (36), the formation of 8-*iso*-$PGF_{2\alpha}$ can be promoted by GSH (figure 1) (37). Thus, assuming that UWPC had increased lung GSH, as demonstrated in liver, the above mechanisms may have resulted in a neutral effect of UWPC in 8-*iso*-$PGF_{2\alpha}$ formation.

Modulation of the formation of relaxing mediators by oxidative stress

In addition to affecting the formation of airway contractile mediators, GSH may influence the formation of airway relaxants. For instance, it is well known that reactive oxygen species, especially superoxide, can substantially diminish the bioavailability of the (airway) smooth muscle relaxant (38), NO (39). Accordingly, several antioxidants can enhance the relaxant effect of NO, although an interference with the inhibitory action of ROS is not proven for all of them (40). As described earlier in this chapter, GSH may not only prevent the breakdown of NO by scavenging ROS, but it can also bind to NO, thus forming GSNO as described by Gaston (10). GSNO is more stable than genuine NO. Thus, both scavenging of ROS and binding of GSH to NO can enhance the bioavailability of NO as a relaxant mediator. PGE_2 was the only relaxant mediator that could be measured reliably in the perfusates of isolated tracheas (chapter 3) and lungs (chapter 5), although results of other attempts (chapter 4) were ambiguous and were, therefore, not included.

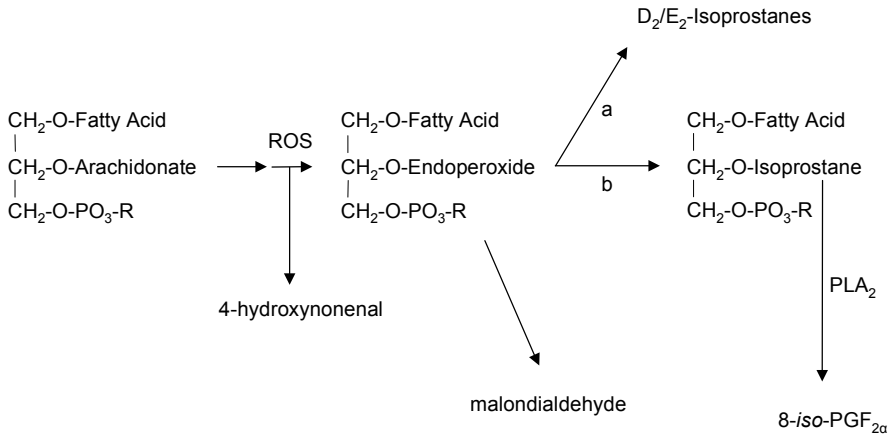

Figure 1. Formation of oxidation products of arachidonic acid. While reaction *a* is a rearrangement, reaction *b* is a reduction step that is promoted by GSH (34). Malondialdehyde and 4-hydroxynonenal are thiobarbituric acid-reactive substances (TBARS). PLA_2, phospholipase A_2.

GSH did not alter PGE_2 levels in the effluent from tracheal tubes, but the data in chapter 5 show that PGE_2 was decreased after UWPC-feeding. Provided that UWPC had enhanced lung GSH levels, these data indicate that GSH decreased PGE_2 formation.

These results are at variance with data from Hempel *et al.*, who have shown that oxidative stress decreased PGE_2 synthesis, and that GSH prevented this decrease (41). In contrast, other data have indicated an inverse relation between PGE_2 synthesis and GSH levels in microsomal fractions from goat lungs (42). Moreover, it has been suggested that PGE_2 production is stimulated by oxidative stress (43) and inhibited by an ROS-scavenger (44). The findings in chapter 5 would be line with the latter three reports.

As mentioned in chapter 5, it is also possible that the altered PGE_2 production was not a direct consequence of altered GSH levels, but rather of the degree of airway contractions. Notably, the severe airway contractions following allergen challenge were paralleled by markedly increased PGE_2 production as compared to the saline-

challenged animals. Similarly, the smaller airway contractions UWPC feeding may have led to less PGE_2 production.

Alteration of smooth muscle responsiveness by oxidative stress

A third mechanism by which oxidative stress may alter smooth muscle contractions is by influencing the responsiveness of smooth muscle to mediators. For instance, it has been reported that the non-enzymatic oxidation product of arachidonic acid, 8-*iso*-$PGF_{2\alpha}$, increases the sensitivity to other contractile agents (45). The mechanism behind this effect is still unclear; however, a study on another lipid peroxidation product, 4-hydroxynonenal, provides a clue. The altered contractile responses to other agents as induced by this compound have been attributed to alkylation of thiol groups (46). Therefore, it is not unlikely that potentiated contractions to other mediators as caused by 8-*iso*-$PGF_{2\alpha}$ were accompanied by alkylation of thiol groups as well. Since GSH potently protects tissues against alkylating agents (47), it may likewise attenuate the effects of 8-*iso*-$PGF_{2\alpha}$ by this action. This may also explain the paradox that the UWPC-induced decrease in lung contractility was paralleled by an increase in *iso*-$PGF_{2\alpha}$. The finding that GSH depletion increased airway contractility to histamine accordingly may be explained by a lack of GSH-mediated protection against alkylation or oxidation of proteins that mediate smooth muscle contractility. Similar mechanisms may underlie the relaxing effects of GSH in the trachea (chapter 3). The finding that K^+-channels mediate these relaxations and the fact that thiol-modifying agents regulate K^+-channel activity suggest that GSH may protect functional sulfhydryl groups in K^+-channels against oxidative modification. The source of the oxidative stress in these experiments may have been the 95% O_2-containing gas mixture used to aerate the Krebs buffer. Indeed, it was recently shown that levels of GSH in rat livers were substantially decreased after 4 h of perfusion with Krebs buffer aerated with 95% O_2, as compared to normoxic gas (21% O_2). Addition of NAC to the buffer prevented the decrease of liver GSH and disturbance of the GSH/GSSG ratio (48). These data suggest that aeration of perfusion buffers with a gas mixture containing 95% O_2 as commonly practiced in organ bath studies can cause unwanted artifacts affecting smooth muscle tension.

Species differences

Whereas respiratory allergen challenge of sensitized guinea pigs induced an unambiguous EAR paralleled by a clear decrease in GSH levels (chapter 4), respiratory challenge of sensitized mice with the same allergen failed to show either of these effects (chapter 6). The presence of a symptomatic EAR in the guinea pig and its absence in the mouse were expected features of the animal models; however, the fact that the presence and absence of an EAR coincided with the presence and

absence of lung GSH depletion was unexpected, and suggests that oxidative stress is a contributing factor of symptomatic EARs. Oxidative stress during allergen challenge, however, is apparently not required for the development of non-specific airway hyperresponsiveness upon allergen challenge, given that both species display this phenomenon. Solid evidence for this notion requires additional studies in guinea pigs in which oxidative stress of the EAR is prevented by exposing the animals to allergen in combination with a GSH supplement.

The fact that asthmatics display a symptomatic EAR may underline the importance of oxidative stress in the pathology of asthma. Indeed, levels of the indicator of oxidative stress, 8-*iso*-$PGF_{2\alpha}$ (49), were found to be increased in exhaled air of asthmatics (50), and 8-*iso*-$PGF_{2\alpha}$ plasma levels correlated with disease severity in mild asthmatics (51). Moreover, in atopic asthmatics, inhaled allergen challenge increased the urinary excretion of 8-*iso*-$PGF_{2\alpha}$ (52). The apparently crucial role of oxidative stress in the guinea pig model, but not in the murine model, of allergic asthma as described in this thesis, suggests that guinea pigs are a better species to study allergic asthma than mice, at least when addressing the role of oxidative stress and therapeutic potential of antioxidants in the context of this disease.

Prophylactic GSH administration

Since asthmatic reactions in humans are accompanied by oxidative stress, asthmatic symptoms may be prevented or relieved by increasing lung levels of antioxidants like GSH. Studies in chapter 6, however, showed that this is not easily achieved. The delivery of aerosols with GSH donors around allergen challenges did not increase tissue levels of GSH in the lungs. These results could be explained by the time point at which GSH levels were assessed in the lungs (24 hours after the last aerosol with a GSH precursor). In that respect, our data are consistent with those of Buhl *et al.*, who showed that the increase of GSH levels in the epithelial lining fluid after application of a GSH aerosol lasted less than two hours (53). In humans, too, only a transient increase in GSH + GSSG was observed in the epithelial lining fluid after a GSH aerosol (54). These studies suggest that GSH or its donors should be delivered relatively frequently to cause a lasting increase in GSH levels. Although some studies have addressed the kinetics of GSH after an aerosol by assessing GSH levels in epithelial lining fluid, the effects on GSH levels in lung parenchyma remain to be determined. This is especially important since most inflammatory cells, and, therefore, probably most of the oxidative stress, are located in the lung parenchyma rather than in the airway lumen. Despite the poor knowledge regarding the kinetics of GSH after the administration of GSH or its donors via aerosols, this mode of administration has yielded some promising results. For instance, GSH

nebulization has decreased non-specific airway contractions in asthmatics (55) and airway obstruction in COPD patients (56). Additionally, a decrease of inflammatory cell activity in idiopathic pulmonary fibrosis patients was demonstrated in a study by Borok *et al.* GSH aerosols in these patients attenuated the increased spontaneous superoxide release by their macrophages (54). These studies reported that administration of thiol compounds had no adverse effects. Only one study has reported bronchoconstriction in mild asthmatics upon administration of GSH by aerosol (57). This study, however, used very high concentrations (0.5 M) of GSH.

Alternative methods to increase lung GSH levels include oral administration of GSH precursors. A GSH precursor that has been used in the context of lung diseases for over 2 decades is the mucolytic, N-acetylcysteine (NAC). Although the airways provide an excellent route for topical administration, NAC is usually given orally (58). However, high oral doses of NAC (300 mg three times per day, or 600 mg once daily), did not increase cysteine concentrations in airway lining fluid (59, 60). As far as known, effects of oral NAC administration on GSH levels in lung parenchyma have not been assessed. It is not impossible that these are increased, since oral administration of NAC was found to increase plasma levels of GSH (59) and since lung tissue appeared capable of utilizing plasma GSH (61, 62). However, there is currently no evidence to prove that this route effectively increases GSH levels in lung parenchyma.

UWPC

An alternative to oral NAC administration to increase tissue GSH levels may be provided by an undenatured whey protein concentrate, UWPC. In guinea pigs, daily feeding UWPC for 24 days increased liver total glutathione levels by 20% (chapter 5). This figure agrees well with results of a study in HIV-infected patients (63), in whom UWPC, given as a food supplement, increased plasma GSH levels by 24%. Similar as in the study in chapter 5, indicators of oxidative stress were not changed in the UWPC-treated HIV-positive patients. This was also found in patients who received a different brand of whey-based food supplement that induced a 45% increase in plasma GSH levels (63). The absence of improved clinical parameters in that study contrasts with a case report describing a woman with obstructive lung disease (64). Upon taking UWPC, her plasma GSH levels increased twofold, and the lung function parameters, FEV_1 and FVC, were improved.

The fact that in guinea pigs a modest increase in GSH levels was associated with a significant and substantial decrease in allergen-induced lung contractility is surprising. The patent application of UWPC claims that it is rich in several vitamins

B, which would promote the reduced state of GSH. If this is true, UWPC would not only enhance GSH levels, but also sustain the reduced state of the molecule. However, there is no literature supporting this notion.

The merits of UWPC in the model described in chapter 5 and its relevance to human asthma, may be more solid when reliable data on GSH and GSSG levels in the lungs will be available. Thus, it should be investigated whether UWPC diminishes the decrease in lung GSH in sensitized guinea pigs after allergen challenge *in vivo*.

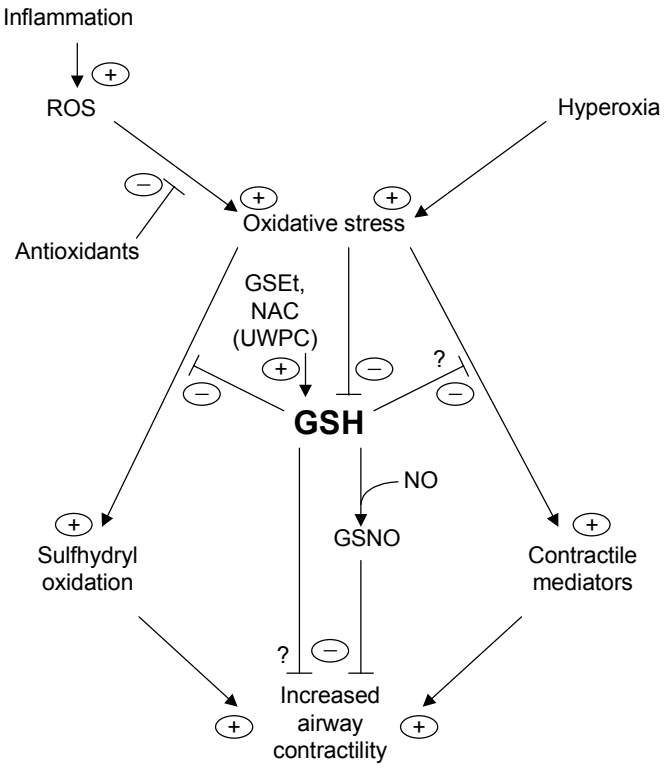

Figure 2. Outline of the major mechanisms regarding GSH as a moderator of airway contractility as addressed in this thesis. Oxidative stress can result either from an inflammatory reaction via the production of ROS *in vivo*, or from hyperoxic conditions in organ baths. The oxidative stress could lead to increased airway contractility via oxidation of critical sulfhydryl groups, increased formation of contractile mediators, and / or other, yet unidentified mechanisms. GSH may interfere with these events and thus prevent or decrease increased airway contractility. Several GSH enhancing compounds, like GSEt, NAC, and possibly UWPC, may therefore have therapeutic potential in asthma.

In conclusion, the data presented in this thesis indicate that GSH protects the airways against excessive constriction, probably by virtue of its antioxidant properties. An outline of the major mechanisms and limitations in scientific evidence as addressed in this thesis is presented in Figure 2. In view of the presence of increased airway contractility and the accumulating evidence of oxidative stress in asthma, the use of GSH to alleviate asthmatic symptoms certainly warrants further research.

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Nederlandstalige samenvatting

Allergisch astma is een longaandoening waaraan wereldwijd 5% van de volwassenen en 10 tot 15% van de kinderen lijden. Een belangrijk kenmerk van allergisch astma is een chronische ontsteking van de luchtwegen. De ontstekingscellen die hierbij betrokken zijn produceren zogenaamde reactieve vormen van zuurstof (afgekort ROS, naar de Engelse term *reactive oxygen species*). Dit zijn zuurstofverbindingen die aggressiever zijn dan moleculair zuurstof en daarom zeer geschikt voor de verdediging van het lichaam tegen ziekteverwekkers. De productie van ROS is dan ook een normaal verschijnsel tijdens een infectie. Het lichaam zelf wordt tegen ROS beschermd door antioxidanten. In bepaalde gevallen brengen ROS toch schade toe aan het eigen weefsel. Dit kan het gevolg zijn van een excessieve productie van ROS door het ontsporen van het afweersysteem, of door een tekort aan antioxidanten; waarschijnlijk treedt vaak een combinatie van beide op. Deze disbalans, die kan leiden tot schade aan en dysfunctie van weefsels en organen, wordt oxidatieve stress genoemd. Er komen steeds meer aanwijzingen dat oxidatieve stress een rol speelt in de pathologie van astma.
Een in het lichaam - en ook in de longen - veelvoorkomend antioxidans is glutathion. Wanneer gereduceerd glutathion (de normale vorm van glutathion) optreedt als beschermer van het weefsel tegen oxidatieve stress wordt het omgezet in geoxideerd glutathion; glutathion wordt dus opgeofferd teneinde het weefsel tegen oxidatie te beschermen. Algemeen wordt de balans tussen gereduceerd en geoxideerd glutathion (GSH resp. GSSG) beschouwd als een indicator voor oxidatieve stress.

Een tweede kenmerk van allergisch astma is een overmatige vernauwing van de luchtwegen (luchtweghyperreactiviteit). De diameter van de luchtwegen kan worden verkleind door autonoom, zogenaamd *glad* spierweefsel. Samentrekking (contractie of constrictie) van glad spierweefsel in de luchtwegen kan teweeggebracht worden door aspecifieke prikkels, zoals koude lucht of mist, of door specifieke prikkels, zoals de ontstekingsmediator histamine. Er wordt vaak een verband gelegd tussen de ontsteking van de luchtwegen en luchtwegconstrictie, maar hoe de twee precies gerelateerd zijn is vooralsnog onduidelijk.

In dit proefschrift is de vraag aan de orde gesteld of de oxidatieve stress als gevolg van luchtwegontsteking verband kan houden met constrictie van de luchtwegen tijdens astma.

Om contracties van de luchtwegen te bestuderen worden vaak stukjes luchtpijp (trachea) uit dieren geïsoleerd en in leven gehouden in een orgaanbad, waarin de spiercontracties bepaald kunnen worden. In hoofdstuk 2 wordt de bevinding beschreven dat het hogere deel van de luchtpijp (het proximale trachea-segment) van de cavia sterker op een contractiele prikkel reageert dan het lagere (distale) segment. Tijdens de zoektocht naar een verklaring voor dit verschijnsel is onderzocht of GSH-spiegels in de proximale trachea verschilden van die in de distale trachea. Dit bleek niet het geval te zijn, zodat het onwaarschijnlijk is dat GSH iets te maken heeft met de differentiële contractiliteit van de proximale en distale trachea.

Dat GSH wel degelijk in staat is om de contracties van de trachea te moduleren blijkt uit hoofdstuk 3. Hierin is beschreven dat het spierweefsel van trachea's in een orgaanbad ontspande (relaxeerde) door blootstelling aan GSH. Dit proces was dosis-afhankelijk - d.w.z. hoe meer GSH, des te meer relaxatie - en kon geremd worden door een remmer van kaliumkanalen. Kaliumkanalen kunnen de polariteit, en daarmee het vermogen tot samentrekken, van spiercellen mede regelen. Een ander mogelijk mechanisme dat is onderzocht, is een interactie van GSH met de door het lichaam zelf geproduceerde gladde-spier-ontspannende stof, stikstofoxide (NO). NO zelf is zeer instabiel, maar door te binden aan GSH kan het zijn levensduur en biologische beschikbaarheid vergroten. De hoeveelheid NO wordt vaak gemeten door het stabiele afbraakproduct van NO, nitriet te bepalen. Inderdaad wezen metingen uit dat nitrietconcentraties in het orgaanbad toenamen op het moment dat GSH aan het orgaanbad werd toegevoegd en de trachea's ontspanden. Het leek er dus op dat de door GSH veroorzaakte relaxaties gemediëerd werden door NO uit de trachea. Dat dit slechts schijn was, bleek uit twee vervolgexperimenten. In de eerste plaats werden de door GSH geïnduceerde relaxaties niet tegengegaan door een remmer van het enzym guanylaatcyclase, dat nodig is voor ontspanning van glad spierweefsel door NO. In de tweede plaats bleek PTIO, een stof die NO bindt en onwerkzaam maakt, ook geen effect te hebben op de spierontspannende werking van GSH. Blijkbaar vond tijdens de in dit hoofdstuk beschreven experimenten wel een interactie tussen GSH en NO plaats, maar mediëerde NO de door GSH teweeggebrachte relaxaties niet. De verhoogde nitrietconcentraties die eerder op betrokkenheid van NO bij de relaxaties leken te wijzen, kunnen verklaard worden met een model dat onlangs in de literatuur gepresenteerd is. Dit model stelt een reactie voor waarin nitriet, in aanwezigheid van GSH en zuurstof, direct gevormd wordt uit GSNO, het reactieproduct van GSH en NO.

Bij astmatici is het weefsel dat de binnenkant van de luchtwegen bekleedt, het zgn. epitheel, vaak beschadigd. Deze beschadiging verklaart een deel van de

hyperreactiviteit van de luchtwegen van astmatici voor contractiele prikkels. Om deze situatie te imiteren, worden vaak experimenten gedaan met stukjes trachea waarvan het epitheel verwijderd is. Om hun vermogen tot samentrekken te bestuderen worden de eerder beschreven orgaanbaden gebruikt. Net als luchtwegen van astmatici zijn epitheelloze trachea-segmenten hyperreactief ten aanzien van contractiele mediatoren; ze contraheren meer dan intacte trachea-segmenten. Het laatste experiment in hoofdstuk 3 toonde aan dat GSH niet alleen trachea's kan relaxeren, maar dat GSH ook de hyperreactiviteit van epitheelloze trachea's voor ca. 50% kan tegengaan.

Uit het bovenbeschreven hoofdstuk blijkt dus dat GSH de luchtwegen kan relaxeren en ze bovendien kan beschermen tegen hyperreactiviteit. In het licht van deze bevindingen is het interessant om te weten of er in de longen van astmatici minder GSH voorkomt dan in die van gezonde mensen. Omdat het nauwelijks haalbaar is om GSH in longweefsel van astmatici te meten, is voor de beantwoording van deze vraag een diermodel voor allergisch astma gebruikt. GSH en GSSG zijn in de longen van cavia's gemeten op het moment dat ze een aanval van allergisch astma ondergingen (hoofdstuk 4). Inderdaad bleek dat GSH-spiegels in de longen van "acuut astmatische" dieren ca. 40% lager waren dan het geval was bij controledieren. Daarbij waren GSSG-spiegels juist verhoogd; in de longen van deze dieren was dus blijkbaar sprake van oxidatieve stress.
In het kader van deze bevindingen werd in dit hoofdstuk verder onderzocht of het toedienen van extra GSH aan de longen van astmatische dieren een aanval van allergisch astma kon verlichten. Om deze vraag te beantwoorden werden vrijgeprepareerde cavialongen doorspoeld met perfusievloeistof, terwijl hun contractie werd gemeten. Wanneer in deze geprepareerde longen een astmatische aanval werd opgewekt, kon een toename in de contractie van de luchtwegen waargenomen worden. Inderdaad waren deze contracties minder hevig wanneer de longen via de perfusievloeistof werden voorzien van extra GSH. Nog meer bewijs voor het belang van GSH in de luchtwegen kwam van een ander experiment in dit hoofdstuk, waarin werd aangetoond dat geïsoleerde longen, afkomstig van cavia's waarin GSH-spiegels kunstmatig verlaagd waren, hyperreactief waren ten aanzien van de contractiele mediator histamine.

Omdat het toedienen van stoffen aan geïsoleerde longen via een perfusievloeistof een handige route is in het laboratorium maar voor astmapatienten niet reëel is, is in hoofdstuk 5 gezocht naar geschikte mogelijkheden om GSH bij astmatici te verhogen. Orale toediening van GSH zelf biedt geen soelaas omdat het de longen niet bereikt. Er bestaan echter voedingssupplementen op basis van wei (een

bijproduct van de kaasbereiding) waaraan een GSH-verhogend effect wordt toegeschreven. In hoofdstuk 5 wordt beschreven hoe zo'n dieet is getest op het vermogen om een astmatische aanval te verlichten in hetzelfde model voor allergisch astma als beschreven in hoofdstuk 4. Inderdaad waren de astmatische contracties in longen van met wei bijgevoerde cavia's minder ernstig dan het geval was bij longen van met een controlevloeistof bijgevoerde dieren. Dat dit te danken was aan een verhoging van GSH-spiegels is waarschijnlijk, omdat de GSH-spiegels in de levers van behandelde dieren hoger waren dan die van controledieren. Om het werkingsmechanisme van het supplement nader te onderzoeken is getracht te bewijzen dat in de longen van met wei gevoerde dieren minder oxidatieve stress optrad dan in de longen van controledieren. Hoewel twee relevante indicatoren aangaven dat de allergische aanval zelf, zoals verwacht, oxidatieve stress deed toenemen, kon de gunstige werking van het voedingssupplement niet teruggevoerd worden op een vermindering ervan. Evenmin kon worden bewezen dat het voedingssuplement leidde tot een verminderde vorming van contractiele mediatoren of een verhoogde vorming van relaxerende mediatoren. Het voedingssupplement lijkt dus een gunstige werking op de ernst van een astmatische aanval te hebben, maar het achterliggende mechanisme is nog onduidelijk.

De bovenbeschreven bevindingen hebben alle betrekking op een model waarin de allereerste allergische aanval werd bestudeerd, terwijl bij astmatici meestal sprake is van *herhaalde* blootstelling aan een allergische prikkel (allergeen). Om die situatie beter te benaderen is in hoofdstuk 6 gebruik gemaakt van een model waarin de luchtwegen van dieren (in dit geval muizen) herhaaldelijk worden blootgesteld aan een allergeen. Bij aldus behandelde dieren zijn de luchtwegen ontstoken en hyperreactief. Deze luchtweghyperreactiviteit wordt aan het eind van het experiment bepaald door oplopende doses van de contractiele stof methacholine te laten inhaleren en vervolgens hun luchtwegfunctie te meten. Met dit model is onderzocht of geïnhaleerd GSH astmatische symptomen kan voorkomen of verminderen. De inhalatie van GSH vond plaats tijdens of vlak voor elke blootstelling aan allergeen, of gelijktijdig met het inhaleren van methacholine. Het bleek dat GSH de luchtweghyperreactiviteit tegenging wanneer het werd geïnhaleerd tijdens de blootstelling aan methacholine, en niet wanneer GSH gegeven werd tijdens blootstelling aan allergeen. Bovendien leek GSH de ontsteking in de luchtwegen niet effectief te bestrijden, want de hoeveelheid ontstekingscellen in het lumen (de holtes) van de luchtwegen was niet verminderd na behandeling met GSH. Interessant was bovendien dat GSH- en GSSG-concentraties in de muizenlong niet verstoord waren tijdens blootstelling aan allergeen. Het lijkt er dus op dat blootstelling van muizen aan allergeen niet leidt tot oxidatieve stress in hun longen, dit in

tegenstelling tot de situatie in de cavialong. Omdat muizen, in tegenstelling tot cavia's, ook geen symptomatische astmatische aanval doormaken tijdens blootstelling aan allergeen, lijkt het erop dat oxidatieve stress en een acute astmatische reactie aan elkaar gerelateerd zijn. Daarnaast blijkt uit dit hoofdstuk dat de cavia geschikter is voor studies naar aspecten van oxidatieve stress in astma dan de muis.

Concluderend wijzen de in dit proefschrift beschreven experimenten erop dat GSH de luchtwegen kan beschermen tegen excessieve contracties zoals die zich bij astma voordoen. De aanwezigheid van luchtweghyperreactiviteit en de toenemende aanwijzingen voor oxidatieve stress bij astma rechtvaardigt nader onderzoek naar het gebruik van GSH ter verlichting van astmatische symptomen.

List of abbreviations

BAL	Broncho-alveolar lavage
BSO	D,R-buthionine-L-sulfoximine
Cys	L-cysteine
EAR	Early asthmatic reaction
E_{max}	Maximum response
EPO	Eosinophil peroxidase
GSEt	Glutathione-ethyl ester
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSSG	Oxidized glutathione
GTN	Glyceryl trinitrate
H_2O_2	Hydrogen peroxide
8-*iso*-$PGF_{2\alpha}$	8-*iso*-Prostaglandin $F_{2\alpha}$
MPO	Myeloperoxidase
NAC	N-acetyl-L-cysteine;
NEM	N-ethyl maleimide
NO	Nitric oxide;
$O_2^{\cdot -}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OVA	Ovalbumin
Penh	Enhanced pause
PGE_2	Prostaglandin E_2
PTIO	2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
ROS	Reactive oxygen species
RSNO	S-nitrosothiol

sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
TBARS	Thiobarbituric reactive substances
TEA	Tetraethylammonium
UWPC	Undenatured whey protein concentrate

Curriculum vitae

Joris Kloek werd geboren op 10 juni 1970 te Utrecht. Na het doorlopen van het Gemeentelijk Gymnasium te Hilversum begon hij in 1988 met de studie Biomedische Wetenschappen aan de Rijksuniversiteit Leiden. De propaedeuse verleende toegang tot de toenmalige kopstudie Bio-Farmaceutische Wetenschappen. Met de onderzoekstage "Prodrugs of 5-aminolevulinic acid for photodynamic therapy: synthesis and evaluation" aan de afdeling Medicinale Fotochemie van het Leiden / Amsterdam Center for Drug Research behaalde hij in 1995 zijn doctoraal. Van 1995 tot eind 1996 was hij als projectmedewerker in dienst van genoemde afdeling. Hierna was hij korte tijd als onbezoldigd gastmedewerker verbonden aan de afdeling Medische Biochemie van de Faculteit Geneeskunde te Leiden. In april 1997 trad hij als assistent in opleiding in dienst van de Faculteit Farmacie van de Universiteit Utrecht. In de disciplinegroep Farmacologie & Pathofysiologie werd in samenwerking met Ing. I. van Ark en onder leiding van dr. N. Bloksma, dr. G. Folkerts, prof. dr. F. De Clerck en prof. dr. F.P. Nijkamp gestalte gegeven aan het onderzoeksproject waarvan de resultaten in dit proefschrift beschreven zijn. Een deel van dit onderzoek werd in 2001 bekroond met een Travel Award Grant van de American Thoracic Society. In 1999 en in 2000 werden delen van het onderzoek uitgevoerd tijdens werkbezoeken aan de laboratoria van dr. B. Gaston (University of Virginia, Charlottesville, Virginia) en prof. dr. J.M. Drazen (Harvard Medical School, Boston, Massachusetts).

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World Asthma Meeting, December 1998, Barcelona, Spain. Glutathione decreases tracheal hyperreactivity caused by epithelial denudation in a perfused organ bath. *Poster presentation*.

95th International conference of the American Thoracic Society, April 1999, San Diego, USA. Glutathione subdues hyperreactivity of the guinea-pig trachea caused by epithelial denudation in a perfused organ bath. *Poster presentation*.

Biology of Nitric Oxide, September 1999, Stockholm, Sweden. Glutathione and other thiols relax guinea pig trachea *ex vivo* and enhance NO-concentrations in the tracheal effluent. *Oral presentation*.

96th International Conference of the American Thoracic Society / Canadian Thoracic Society. May 2000, Toronto, Canada. Relaxing effects of glutathione and other thiols in guinea pig trachea *ex vivo*: interactions with nitric oxide. *Poster presentation*.

Biology, Chemistry and Therapeutic Applications of Nitric Oxide. June 2000, San Francisco, USA. Thiol-induced decrease of guinea pig tracheal tone *ex vivo* is associated with enhanced nitrite levels but not inhibited by PTIO. *Poster presentation*.

97th International Conference of the American Thoracic Society May 2001, San Francisco, USA. Glutathione modulates non-specific and allergen-induced airway responsiveness in the guinea pig. *Poster presentation*.

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Allereerst wil ik mijn promotores, Frans en Fred, bedanken; niet alleen voor het vertrouwen, maar ook voor jullie flexibiliteit. Het onderzoeksproject maakte na enkele maanden een niet te verwaarlozen accentverschuiving door: van het belang van arginine naar het belang van glutathion voor de luchtwegen. Jullie hebben dat proces veeleer aangemoedigd dan ontmoedigd. Hartelijk dank daarvoor.
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Ben, your very kind hospitality when I visited your lab was terrific. My stay was a great opportunity for me to learn more about nitrosothiols in an excellent atmosphere (even though the project has drifted somewhat away from those compounds...). It is an honor for me that you are a member of the *beoordelingscommissie* (try your best German on that one!).
Craig and professor Drazen, thank you both very much for my stay in your lab, and for providing the opportunity to learn and work with the perfused lung set up.

Binnen de afdeling hebben velen me op uiteenlopende manieren geholpen. Door mensen met naam en toenaam te noemen zou ik anderen misschien tekortdoen; bedankt dus, Fijne Farmacologie Familie, voor congresplezier, begripvolle oren, tips betreffende de wetenschap of andere zaken, de vrijdagborrels, e-mails toen ik in den vreemde verbleef, en nog veel meer.

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Stellingen behorende bij het proefschrift
***Glutathione and airway function* door Joris Kloek**

1. Het gebruik van meerdere trachea-segmenten van hetzelfde dier voor de bestudering van luchtwegreactiviteit met het oogmerk om proefdier-besparend te werken, kan contraproductief uitpakken en verkeerde conclusies in de hand werken.
   Dit proefschrift

2. Voor de bestudering van oxidatieve stress bij astma is de cavia een geschikter proefdier dan de muis.
   Dit proefschrift

3. Het meten van totaal glutathion in de broncho-alveolaire lavage levert onvoldoende informatie om een uitspraak te doen over de gevolgen van oxidatieve stress in de longen voor de glutathion-huishouding.
   Smith, L.J., M. Houston, and J. Anderson. 1993. Increased levels of glutathione in bronchoalveolar lavage fluid from patients with asthma. *Am Rev Respir Dis* 147:1461.

4. De invloed van zuurstofspanning op antioxidant systemen van organen in orgaanbaden verdient nadere bestudering.
   Miralles, C., X. Busquets, C. Santos, B. Togores, S. Hussain, I. Rahman, W. MacNee, and A.G. Agusti. 2000. Regulation of iNOS expression and glutathione levels in rat liver by oxygen tension. *FEBS Lett* 476:253.

5. Het schrijven van een projectvoorstel is voor een promovendus minstens even leerzaam als het uitvoeren ervan.

6. Kloeks zesde stelling: uiteindelijk toch houdbaar.
   J.J. Kloek (1985) Over Werther geschreven…
   J. Kloek (1941) Het begrip regressie

7. De kwalificatie 'typisch Nederlands', in misprijzende zin gebruikt, wordt met name gebezigd door mensen die de aandacht willen vestigen op de vermeende breedte van hun horizon.

8. De binnenvaart belichaamt al eeuwen de beste Betuwelijn.

9. Het stelselmatig maken van verre reizen om andere culturen te ontmoeten ondergraaft op den duur de reden van deze reizen.

10. Het grote aantal witte jassen in ziekenhuiskantines suggereert dat veel artsen meer waarde hechten aan status dan aan hygiëne.