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

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Kloek, Joris, Ingrid van Ark, Nanne Bloksma, Fred De Clerck, Frans P. Nijkamp, and Gert Folkerts. Glutathione and other low-molecular-weight thiols relax guinea pig trachea ex vivo: interactions with nitric oxide? Am J Physiol Lung Cell Mol Physiol 283: L403–L408, 2002. First published April 12, 2002; 10.1152/ajplung.00376.2001.—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) (Gaston B. Biochim Biophys Acta 1411: 323–333, 1999; Kelm M. Biochim Biophys Acta 1411: 273–289, 1999; and Kharitonov VG, Sundquist AR, and Sharma VS. J Biol Chem 270: 28158–28164, 1995) and the high (millimolar) concentrations of GSH in trachea epithelium (Rahman I, Li XY, Donaldson K, Harrison DJ, and MacNee W. Am J Physiol Lung Cell Mol Physiol 269: L285–L292, 1995). GSH and other thiols (1.0–10 mM) dose dependently decreased the tension in isolated guinea pig tracheas. Relaxations by GSH were paralleled with sevenfold increased nitrite levels (P < 0.05) in the tracheal effluent, suggesting an interaction between GSH and NO. However, preincubation with a NO scavenger did not reduce the relaxations by GSH or its NO adduct, S-nitrosoglutathione (GSNO). Inhibition of guanylyl cyclase inhibited the relaxations induced by GSNO, but not by GSH. Blocking potassium channels, 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. These data indicate that GSH plays a role in maintaining tracheal tone. The mechanism is probably an antioxidative action of GSH itself rather than an action of NO or GSNO.

nitrosothiols; epithelium; potassium channels; guanylyl cyclase

Airway hyperreactiveness is a key feature of several lung diseases. It is often associated with epithelial damage as a consequence of inflammatory processes (22). 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 (15). 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 (4, 35). 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 (16, 20, 23). 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 (29). RSNOs are also produced by direct binding of nitrosourea ions to thiols (28). RSNOs can be regarded as stable pools of NO (18) and are themselves directly implicated in relaxing airway smooth muscle (24, 37). 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 nonprotein thiols and plays a pivotal role in a variety of enzymatic and nonenzymatic reactions that protect tissues against oxidative stress (32). In view of the antioxidant role of GSH and widespread 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. Lung epithelial cells can be estimated to contain 10 mM GSH (39), and the epithelial lining fluid in the lungs contains 400 μM GSH, 100-fold higher than GSH levels in plasma (9).

In antioxidative reactions, GSH is converted into its oxidized form, glutathione disulfide (GSSG), that in its turn is enzymatically reduced into GSH to maintain a physiological redox balance. Under normal conditions, 95–99% of total GSH in the body is present in the reduced form (32). However, inflammatory diseases like asthma are associated with oxidative stress that

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places a large burden on the GSH pool (3, 11, 12). 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 (27).

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. Because GSH-induced relaxations were indeed paralleled by a rise in nitrite levels in the buffer, we investigated whether guanylyl cyclase and potassium channels mediated these relaxations, since both guanylyl cyclase (6, 7, 26) and potassium channels (1, 25) are known play a role in NO-induced smooth muscle relaxation. Finally, the physiological 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 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 pentobarbital sodium (Nembutal; 0.6 g/kg ip body wt). Tracheas were dissected free of connective tissue and blood vessels, isolated, and divided into proximal and distal parts. Where indicated, the epithelial layer was removed from the tracheal segments as described earlier (14). Proper removal of the epithelium without causing damage to the underlying tissues was confirmed by light microscopy. Tracheas were mounted in perfused organ baths according to a modified method of Pavlovic et al. (36). The organ baths contained Krebs buffer (pH 7.4) of the following composition (mM): 118.1 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 25.0 NaHCO3, 1.2 K2HPO4, and 8.3 glucose. 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% CO2 in O2 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 analog-to-digital converter, delivering digital signals to a computerized setup. The sampling frequency was 35 Hz. The setup allowed continuous sampling, online 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 pre-tensions 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 (0.1–10 mM) of GSH, L-cysteine (Cys), or N-acetyl-L-cysteine (NAC). As a control, the nonthiol 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.** For NO measurements, nitrite was assayed as a stable and representative breakdown product of NO formed enzymatically or NO released from RSNOs (28). Samples of 100 μl of tracheal effluent were collected just before 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). The sensitivity of the NO analyzer was 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 (33).

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) (2). PTIO was added to the luminal buffer after the 60-min equilibrium period at a final concentration of 100 μM. Twenty minutes after adding PTIO, tracheas were relaxed by intraluminal addition of GSH, S-nitrosoglutathione (GSNO), or glyceral trinitrate (GTN). The compounds were given in concentrations that evoke a submaximal 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]quinolin-1-one (ODQ; 50 μM) (21) or the nonselective potassium channel blocker tetraethylammonium (TEA; 20 mM) (31) was applied to both the mucosal and the serosal buffer. Controls received the vehicle of ODQ (DMSO; 0.25% [vol/vol] final concentration). After 20 min of incubation, GSH (5 mM) or GSNO (100 μM) was added to the luminal buffer without removing TEA or ODQ, and relaxations were recorded.

A 100-μl sample of the luminal perfusate of tracheas was taken before and after addition of GSH to be assayed for prostaglandin E2 (PGE2) content. PGE2 was quantified with an enzyme-linked immunoassay (Amersham, Roosendaal, The Netherlands) according to the manufacturer’s instructions.

**Effect of GSH on tracheal responsiveness to histamine.** Epithelium-denuded tracheas were preincubated intraluminally with GSH at a concentration of 1.0 mM or with saline (controls). After 40 min, optimum tension (2.0 g) was readjusted mechanically, and contractions were measured to increasing concentrations (10-5 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). TEA was purchased from Merck (Darmstadt, Germany). 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 milligrams of tension on top of the 2.0-g baseline tone. Data are expressed as means ± SE. For most experiments, significance calculations were performed using the two-tailed Student’s t-test. Significance was accepted as P < 0.05.
Student’s t-test. However, Wilcoxon’s signed-rank test was used for assessing the statistics of the nitrite measurements, while a repeated measures analysis with a least significance differences 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 (Fig. 1A). Relaxations started within seconds after administration of thiols (Fig. 1C 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 −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. GSH-induced relaxations were more extensive in denuded tissues than in tissues with intact epithelium (Fig. 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.

There were no obvious differences in kinetics among the different thiols.

Mechanisms of GSH-induced tracheal relaxations. Because 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. After the 60-min stabilization period, i.e., just before addition of GSH, nitrite levels in the tracheal effluent were 0.9 \( \mu \)M (95% confidence interval, 0.62–1.2), whereas immediately after addition of 10 mM GSH, levels increased significantly (\( P < 0.05 \)) to 7.9 \( \mu \)M (95% confidence interval 4.3–11.4; Fig. 2), 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 (2) was added to the perfusion buffer before administration of GSH, GSNO, or GTN. PTIO did not significantly alter GSH- or GSNO-induced relaxations (Fig. 3). Because 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 (17) did not affect the ability of GSH to cause tracheal relaxation (Fig. 4), suggesting that the relaxing effect of GSH was not due to a rise in cGMP levels.

An alternative target of smooth muscle relaxation by NO is potassium channels. Upon treatment with the

Fig. 1. A: relaxing effects of the thiol compounds glutathione (GSH; ●), N-acetyl-L-cysteine (▲), and L-cysteine (●), and of the control compound, valine (○), in guinea pig tracheas without epithelium. B: inhibitory influence of the epithelium on the relaxing effects of GSH (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. C: a 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 M) are indicated.

Fig. 2. 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.
nonspecific potassium channel inhibitor TEA (31), the GSH-induced relaxations were almost completely abolished (Fig. 4), suggesting that potassium channels mediate thiol-induced relaxations. Furthermore, 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 (Fig. 4).

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. This hyperresponsiveness upon removal of epithelium has been reported earlier (34, 42). Preincubation with 1.0 mM GSH significantly attenuated the hyperreactivity in tracheae without epithelium by 31.6 ± 9.46% in terms of maximum response to histamine but did not change responsiveness of tracheae with intact epithelium (Fig. 5).

**DISCUSSION**

In this study, we have shown that GSH and other thiols reduced the smooth muscle tone of epithelium-denuded guinea pig tracheae in perfused organ baths. Relaxations up to 65–70% below baseline tension were induced by 10 mM GSH or NAC, the highest concentration tested (Fig. 1A). This concentration may be physiologically relevant because GSH is estimated to be present at this concentration in epithelial cells (39). 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 (10, 40, 43). In addition, the almost instantaneous relaxation induced by the thiols points to an extracellular rather than an intracellular mechanism. l-Valine failed to relax tracheae at any concentration, suggesting that induction of relaxation requires a sulfhydryl group and was not due to nonspecific 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 tracheae tested (\(n = 6\)), but it relaxed some tracheae (\(n = 3\)) to the same extent as GSH. The latter observation is hard to explain. Because relatively high concentrations were required for relaxation, instantaneous reduction of GSSG to GSH by the latter tracheae seems unlikely.

Relaxations were more pronounced in epithelium-denuded tracheae than in intact tissues (Fig. 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 additional GSH can only have a limited effect.
Interestingly, GSH-induced relaxations in epithelium-denuded tissues were paralleled with a sevenfold rise in nitrite levels in the tracheal effluent (Fig. 2), suggesting release of NO by GSH (28). In view of the absence of the epithelium, the increase of nitrite levels has to be derived from sources other 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 (5). It is doubtful, however, whether GSH caused relaxation by releasing genuine NO, in view of the effect of the free NO scavenger PTIO (2). This agent failed to inhibit relaxation by GSH, while it clearly inhibited relaxation by the genuine NO donor GTN (13) (Fig. 3). 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 subepithelial tissues. Those are likely to have been formed there before removal of the epithelium, because, once produced, NO can diffuse to neighboring cells and nitrosylate protein and nonprotein thiols via nitrosylating agents, like dinitrogen trioxide (29) and nitrosyl ions (28). GSH would then interact with tissue RSNOs to yield nitrite without the appearance of NO as an intermediate (41). It is also known that GSH forms GSNO in the presence of protein RSNOs (38). 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 (21), abolished the relaxing effect of exogenous GSNO, but not of GSH.

To see whether PGE$_2$ would mediate the GSH-induced relaxations, this major relaxant prostaglandin was measured in the perfusate of epithelium-denuded tracheas before and during the GSH-induced relaxation. PGE$_2$ levels remained unaltered on 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 nonspecific potassium channel inhibitor TEA were investigated. TEA almost completely abolished trachea relaxation by GSH (Fig. 4), suggesting that GSH can activate particular potassium 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 potassium channels, while sulfhydryl reducing agents, like dithiothreitol and GSH, were shown to reverse inactivation or to cause their activation (8, 44). Further studies are needed to point out which potassium channel is involved in the observed relaxation and whether activation of that channel is regulated by sulfhydryl modification.

Interestingly, potassium channel inhibition also inhibited GSNO-induced tracheal relaxation. Hence, in guinea pig trachea, GSNO apparently induces relaxation through cGMP-induced potassium channel activation. cGMP-dependent activation of calcium-activated potassium channels by RSNOs was reported earlier in rabbit coronary artery smooth muscle (19).

In addition to causing tracheal relaxation, GSH also counteracted histamine-induced contraction in epithelium-denuded tracheas (Fig. 5). The more pronounced activity in epithelium-free tracheas is probably due to the same reasons pointed out above. The finding, however, is relevant because airway hyperresponsiveness is associated with sloughing of the epithelial layer (22).

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 antioxidative 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 (Fig. 5) suggests that replenishment of GSH in the airways might have therapeutic potential at physiological concentrations. Thiol replenishment in asthma has only been described once. Nebulization of GSH in mild asthma caused bronchoconstriction rather than a relief of symptoms (30), but this adverse effect may have been caused by the supraphysiological 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.

REFERENCES


