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 ($E_{\text{max}}$ 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 ($E_{\text{max}}$ 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 E₂ (PGE₂) (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), CaCl₂ (2.5), MgSO₄ (1.2), NaHCO₃ (25.0), K₂HPO₄ (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% CO₂ in O₂ 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 HClO₄ 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.
Chapter 2

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; $E_{\text{max}}$ 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.
Responsiveness of proximal and distal trachea

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](image)

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 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 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.
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.

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<tr>
<td>GSH</td>
<td>485 ± 22.3</td>
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<td>GSSG</td>
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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.
References


