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Regulation of Sympathetic and Parasympathetic Receptor Responses in the Rat Trachea by Epithelium: Influence of Mechanical and Chemical Removal of Epithelium

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Abstract—Removal of the epithelial layer of rat tracheal tissue did not affect the methacholine-induced contraction of the tracheal smooth muscle, but attenuated the (−)-isoprenaline induced relaxation (expressed as percentage of the methacholine contraction). In this way the epithelial layer seemed to play a role in the maintenance of an autonomic balance between sympathetic and parasympathetic receptor responses. Incubation of rat tracheal tissue with cumene hydroperoxide (3×10^{-5}–10^{-3} M) resulted in a dose-dependent destruction and (partial) removal of the epithelial layer. Cumene hydroperoxide diminished muscarinic receptor responses of the rat trachea. Moreover, the autonomic balance between muscarinic and β-adrenoceptor responses was affected. The effects of cumene hydroperoxide on receptor responses were more pronounced after epithelium removal. The protective role of the epithelial layer of pulmonary tissue against oxidative stress has therefore been emphasized.

Asthma is a disease characterized by pulmonary inflammation, reversible airway obstruction and a hyperreactivity of the lungs to several stimuli such as histamine, methacholine, cold air or exercise. During the inflammatory reaction, macrophages and mast cells become activated and release chemotactic products. Neutrophils and eosinophils are then attracted to the inflamed pulmonary tissue (Chung 1986). Activated macrophages, neutrophils and eosinophils produce superoxide anions by the membrane bound enzyme NADPH oxidase, which are converted to other reactive oxygen species (ROS) such as hydrogen peroxide, the hydroxyl radical, singlet oxygen and hypochlorous acid (Southern & Powis 1988a, b). ROS are able to destroy DNA and proteins, and can induce the process of lipid peroxidation (Southern & Powis 1988a). During lipid peroxidation (the stepwise breakdown of polyunsaturated fatty acids) ketones, aldehydes and lipid (oxygen) radicals are formed. The lipid (oxygen) radicals also damage DNA and proteins and promote lipid peroxidation (Vaca et al 1988). ROS have been reported to be involved in the genesis of lung damage, not only in asthma but also in the adult respiratory distress syndrome or after paraquat intoxication, bleomycin administration or mineral dust inhalation (Heffner & Repine 1989).

Recently we investigated the effects of hydrogen peroxide on muscarinic and β-adrenergic receptor responses and observed an imbalance between the sympathetic and parasympathetic receptor responses in favour of the parasympathetic receptor responses (Kramer et al 1987). We have now examined the effects of an organic peroxide, cumene hydroperoxide, on rat tracheal muscarinic and β-adrenoceptor responses. Since asthmatic lungs can show shedding and/or loss of the epithelial layer (Houston et al 1953), we have also determined the role of the epithelial layer in protection against cumene hydroperoxide-induced damage on muscarinic and β-adrenoceptor responses.

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Materials and Methods

Materials

Methacholine chloride, (−)-isoprenaline hydrochloride and cumene hydroperoxide were obtained from Sigma, St. Louis, MO, USA; azocarmine G and aniline blue from E. Merck, Darmstadt, Germany, and orange G were from BDH Chemicals Ltd, Poole, UK. All other chemicals of reagent grade were obtained from either E. Merck, Darmstadt, Germany or J. Baker Chemicals, Deventer, The Netherlands.

Male Wistar rats (Harlan CPB, Zeist, The Netherlands), 200–250 g, were used.

Pharmacological activities

Rats were killed by a blow on the head, and bled. The trachea was rapidly excised and mounted in Krebs buffer with the following composition (mM): NaCl, 117·5; KCl, 5·6; $MgSO_4$, 1·2; $CaCl_2$, 2·5; NaH_2PO_4, 1·28; $NaHCO_3$, 25·0; glucose, 5·5, continuously gassed with a mixture of 5% CO_2 and 95% O_2 in order to maintain oxygen tension and a pH of 7·4 at 37°C. The trachea was dissected free from surrounding tissue and cut in a longitudinal direction opposite the muscle. To remove the epithelial layer the trachea was gently rubbed 4 times with a cotton swab. Removal of the epithelium was checked by histological examination, as described below. The trachea was further prepared according to the method of Timmerman & Scheffer (1968). Each preparation contained four tracheal rings in succession. The dose response curves of the tracheal strips at 37°C were isotonically recorded with a passive force of 0·5 g. After a 60 min washing period with four intermediate changes of buffer solution, cumulative doses of either methacholine or (−)-isoprenaline (precontracted with methacholine, 3×10^{-7} M) were added to the organ bath and the contraction or relaxation of the tracheal

strip was recorded. Between each dose-response curve the tracheae were washed for 35 min with 8 intermediate changes of Krebs buffer solution. After three dose response curves and a washing procedure the tracheal preparations were incubated for 30 min with a single dose of cumene hydroperoxide. After a washing period, methacholine or (−)-isoprenaline dose response curves (precontracted with methacholine, 3×10^{-7} M) were recorded.

Microscopic preparations

Tracheae were obtained as described above and mounted in Krebs buffer. After a 60 min washing period the preparations were incubated for 30 min with different concentrations of cumene hydroperoxide. Subsequently, a 35 min washing procedure was applied and the strips were fixed in 25% formalin, 5% concentrated acetic acid in a saturated picric acid solution. Tissue was embedded in paraffin, sectioned at 7 μm and stained with azocarmine G, aniline blue and orange G (Gurr 1962).

Statistics

All data are the mean of four experiments which were performed at least in duplicate. Results were evaluated according to Student's *t*-test for unpaired samples.

Results

Removal of the epithelial layer had no effect on the methacholine-induced muscarinic receptor response of the rat trachea (Fig. 1). The (−)-isoprenaline-induced tracheal relaxation (after precontraction with methacholine, 3×10^{-7} M) was attenuated after removal of the epithelial layer (Fig. 2). In preparations with an intact epithelial layer, the maximal relaxation induced by (−)-isoprenaline was 75·4 ± 3·7% relative to the precontraction. In epithelial-free preparations the maximal relaxion to (−)-isoprenaline was 48·3 ± 6·0%. Removal of the epithelium did not affect the −log EC50 for (−)-isoprenaline (Fig. 2). Apparently removal of epithelium of the rat tracheal smooth muscle results in an attenuation of the β-adrenergic receptor response.

As listed in Table 1, 30 min incubation with cumene hydroperoxide (10^{-4} M) with normal (intact epithelium) rat tracheal preparations resulted in a decrease in the −log EC50 for methacholine. Additionally, after incubation with cumene hydroperoxide (3×10^{-4} M), there was a decrease in the maximal contraction to 37·8 ± 13·5% compared with control. After a 30 min incubation with cumene hydroperoxide (1 mM), methacholine did not contract the smooth muscle. Addition of cumene hydroperoxide up to 1 mM had no effect on the basal tonus of the rat tracheal smooth muscle.

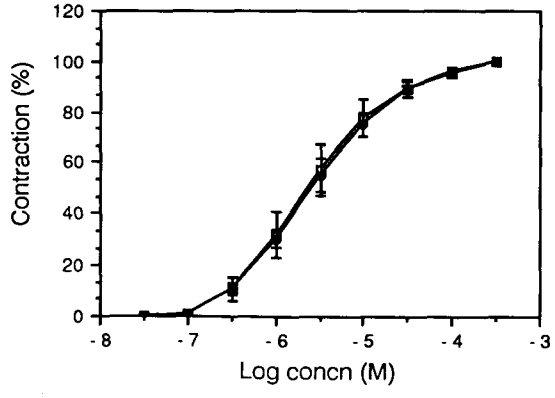

FIG. 1. Effect of epithelium removal on the methacholine-induced contraction of the rat trachea. (□) with epithelium, (◆) without epithelium.

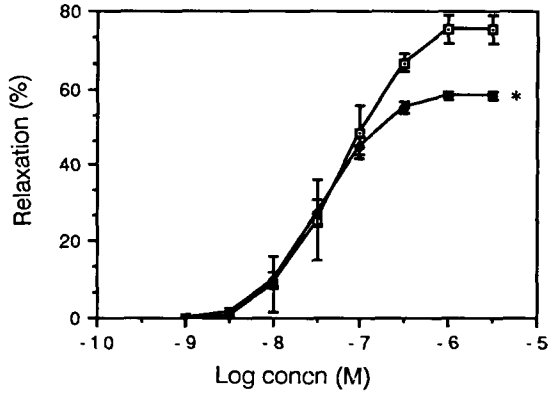

FIG. 2. Effect of epithelium removal on the (−)-isoprenaline-induced relaxation of rat tracheal strips after precontraction with methacholine (3×10^{-7} M). (□) with epithelium, (◆) without epithelium.

As depicted in Table 1, we also found a decreased responsiveness of the epithelium-free tracheal strips to methacholine after cumene hydroperoxide pretreatment. Cumene hydroperoxide also decreased the −log EC50 for methacholine. A significant decrease of maximal effect to 83·7 ± 4·2% was seen after a cumene hydroperoxide (10^{-4} M) incubation. A further decrease in the maximal effect to 17·2 ± 10·2% was seen after cumene hydroperoxide (3×10^{-4} M). As with the intact tissues, in the epithelium-free preparations, we did not observe a response to methacholine after incubation for 30 min with cumene hydroperoxide (1 mM).

Incubation with cumene hydroperoxide, either 3×10^{-5} or 3×10^{-4} M, did not alter the −log EC50 or maximal relaxing effect of (−)-isoprenaline in the tracheal strips with intact epithelium (Table 2). The (−)-isoprenaline-induced maximal relaxation (compared with the precontraction) of epithelium-free preparations increased after incubation with cumene hydroperoxide, either 3×10^{-5} or 3×10^{-4} M (68·9 ± 4·6 and 69·1 ± 5·8, respectively).

Histological stainings (Fig. 3A–D) indicated that incubation with 10^{-4} M cumene hydroperoxide results in a destruction of the basal membrane, and after incubation with higher concentrations of cumene hydroperoxide a loss of the epithelial layer was observed.

Discussion

A reversible airway obstruction, pulmonary inflammation and a hyperreactivity to several stimuli, such as histamine, methacholine, cold air or excercise, are features of asthmatic disease (Houston et al 1953; Chung 1986). Ariëns (1987) stated that the clinically observed hyperreactivity of asthmatic lungs is the result of the observed hypersensitivity to agents (increase in −log EC50) and/or hyperreactivity

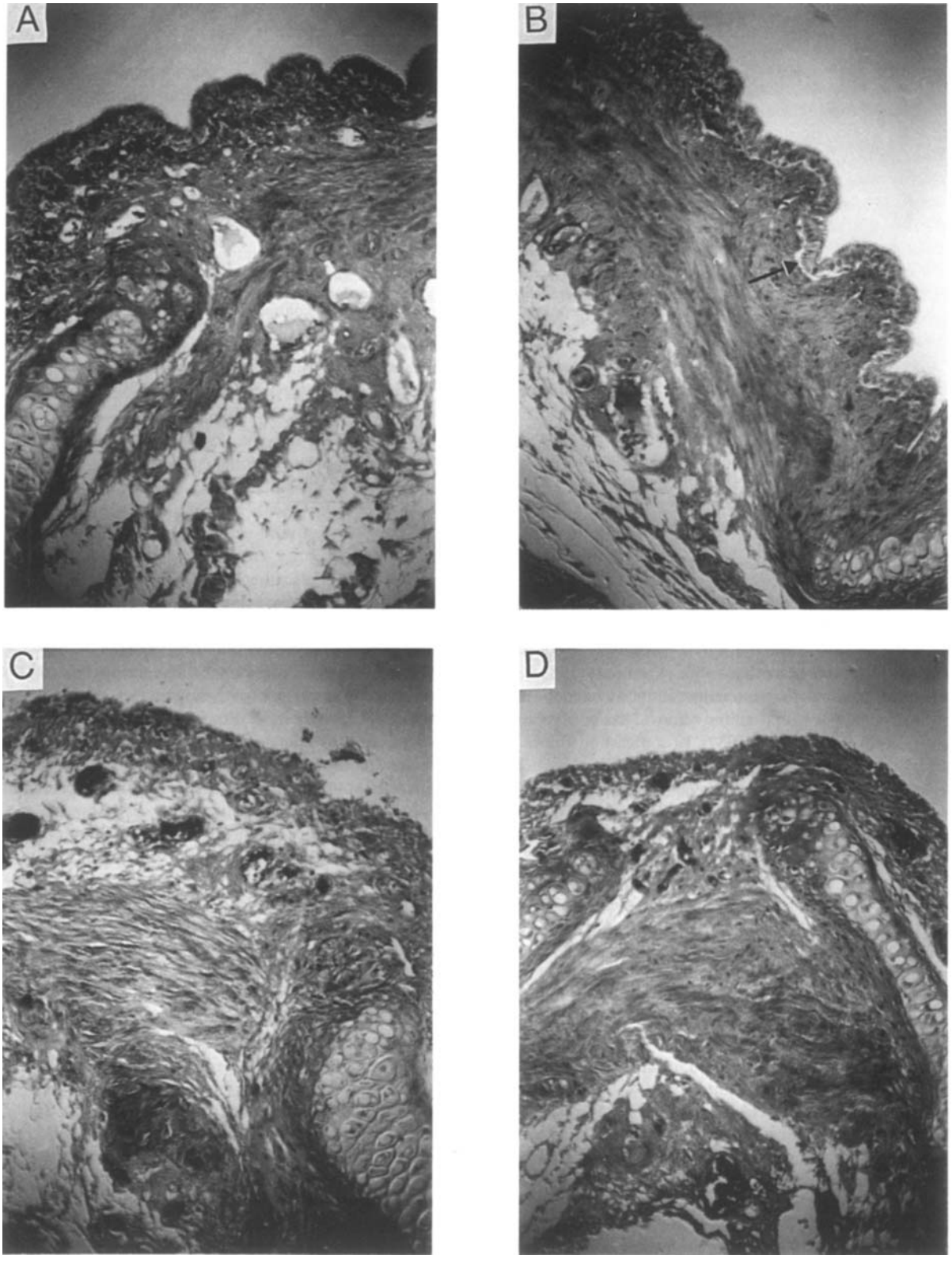

FIG. 3. Histological stainings (200 ×) of rat tracheal tissue after pretreatment with different concentrations of cumene hydroperoxide. A. Trachea with intact epithelium. B. Destruction of the basal membrane (indicated by the arrow) after cumene hydroperoxide incubation (10^{-4} M). C. Partial removal of the epithelial layer after incubation with cumene hydroperoxide (3×10^{-4} M). D. As C (10^{-3} M).

Table 1. The effects of different concentrations of cumene hydroperoxide on the −log ED50 and E_{max} values (±s.e.m.) of methacholine dose response curves of rat tracheal strips with or without epithelium.

Concn of cumene hydroperoxide	With epithelium		Without epithelium	
	−log EC50	E_{max} (%)	−log EC50	E_{max} (%)
Control	5·56±0·12	98·5±1·1	5·62±0·18	99·8±1·7
3×10^{-5} M	5·52±0·10	89·4±5·6	5·70±0·11	97·2±15·7
10^{-4}M	5·03±0·08*	83·7±10·8	5·17±0·05*	83·7±4·2*
3×10^{-4}M	5·19±0·12*	37·8±13·5*	5·24±0·06*	17·2±10·2*
10^{-3}M	ND	0*	ND	0*

* $P<0·05$. ND: not detectable.

Table 2. The effects of different concentrations of cumene hydroperoxide on the β-adrenergic receptor response of the rat tracheal strip, precontracted with methacholine (3×10^{-7} M). The −log EC50 and E_{max} values (±s.e.m.) are given.

Concn of cumene hydroperoxide	With epithelium		Without epithelium	
	−log EC50	E_{max} (%)	−log EC50	E_{max} (%)
Control	7·43±0·22	75·4±3·7	7·59±0·08	48·3±6·0
3.10^{-5} M	7·58±0·06	73·3±5·2	7·62±0·04	68·9±4·6*
$3\cdot10^{-4}$ M	7·41±0·06	65·0±8·5	7·37±0·09	69·1±5·8*

* $P<0·05$.

(increase in maximal response) of the pulmonary smooth muscle. In guinea-pig, dog and man, removal of the epithelial layer of the airways resulted in a hypersensitivity to contracting agents histamine or methacholine (Vanhoutte 1988a). An attenuation of the (−)-isoprenaline-induced relaxations of the dog bronchi due to epithelium removal has been reported (Stuart-Smith & Vanhoutte 1987). In contrast, in guinea-pig tracheal tissue, the (−)-isoprenaline-induced relaxation is increased after epithelium removal (Farmer et al 1986), whereas the relaxation to (−)-isoprenaline is not altered after epithelium removal from human bronchi (Aizawa et al 1988). The epithelium plays a modulating role in the response of the smooth muscle to contracting or relaxing agents. The epithelial layer may act as a diffusion barrier, or extraneuronal uptake of agents may occur in the epithelium. An epithelium-derived relaxing factor (EpDRF) has also been proposed which is continuously released by airway epithelial cells. The nature of this EpDRF is still unknown (Vanhoutte 1988b; Goldie et al 1990).

In our experiments we observed that removal of the epithelium from the rat trachea did not result in a significant change of the methacholine-induced smooth muscle contraction (as shown in Fig. 1), whereas the (−)-isoprenaline-induced relaxation (relative to precontraction with methacholine, 3×10^{-7} M) of the smooth muscle was attenuated after epithelium removal (Fig. 2). These effects of rat tracheal epithelium removal are reported for the first time. These observations indicate that the epithelial layer has a role in the maintenance of the balance between parasympathetic and sympathetic receptor responses in rat pulmonary tissue.

In asthmatics, epithelial destruction coexists with the increased bronchial reactivity (Laitinen et al 1985). Although no correlation was seen between epithelial destruction and bronchial hyperreactivity, it is generally stated that the clinically observed bronchial hyperreactivity of asthmatic lungs could be (partly) due to the destruction or dysfunction of the bronchial layer (Vanhoutte 1988a).

During inflammation, reactive oxygen species (ROS) are produced by macrophages, monocytes, neutrophils and eosinophils (Southern & Powis 1988a, b). Calhoun et al (1987) found an increased superoxide anion release from alveolar macrophages in symptomatic asthmatics. Moreover Meltzer et al (1989) and Postma et al (1988) correlated the measured bronchial reactivity in-vivo of asthmatic patients and patients with chronic obstructive pulmonary disease (COPD) with the ability of blood neutrophils from these patients to produce ROS. They found a correlation between bronchial hyperreactivity and the superoxide anion production of blood neutrophils. Hyperreactivity induced by anaphylaxis or leukotriene D_4 of guinea-pig tracheal segments in-vitro can be blocked by the addition of superoxide dismutase, which indicates that superoxide anions may cause airway hyperreactivity (Weiss & Bellino 1986a, b). These observations suggest that ROS could be (partly) involved in the genesis of bronchial hyperreactivity in-vivo.

Organic hydroperoxides (such as cumene hydroperoxide) are homolytically cleaved in the presence of iron, resulting in the formation of hydroxyl radicals, alkoxy and peroxy radicals (Hunt et al 1988; Davies 1989). We incubated rat tracheal tissue with different concentrations of cumene hydroperoxide (10^{-4}, 3×10^{-4} and 10^{-3} M) for 30 min and made histological stainings of these tracheal strips. As shown in Fig. 3, the basement membrane was destroyed (10^{-4} M) which resulted in the (partial) removal of the epithelial layer from the trachea under influence of cumene hydroperoxide.

Since mechanically removed epithelium of the rat trachea causes an attenuation of (−)-isoprenaline-induced relaxation (as shown here), we anticipate the (−)-isoprenaline

response to be diminished after cumene hydroperoxide pretreatment (which also destroys the epithelium) relative to the methacholine induced precontraction.

We first studied the effects of cumene hydroperoxide on the muscarinic receptor response and the role of the epithelial layer in protection against cumene hydroperoxide. The muscarinic receptor response of the epithelium-free tracheal strip was more susceptible to cumene hydroperoxide treatment than the normal tracheal strip with an intact epithelial layer. Cumene hydroperoxide (10^{-4} M) exposure of the epithelium-free trachea, not only gave a hyposensitivity to methacholine, but also a significant decrease of maximal effect (Table 1) was observed, while with normal trachea only a significant decrease of $-\log$ EC50 for methacholine was observed (after treatment with the same concentration of cumene hydroperoxide). We therefore concluded that the tracheal epithelium protects against cumene hydroperoxide-induced damage. These observations are in agreement with experiments performed by Rhoden & Barnes (1989) who reported that epithelial removal potentiated the hydrogen peroxide-induced contraction of the guinea-pig trachea.

Incubation of rat tracheal strips (intact epithelium) with cumene hydroperoxide (3×10^{-5} or 3×10^{-4} M) did not change the maximal effect or the $-\log$ EC50 of (−)-isoprenaline induced relaxation after precontraction with methacholine (3×10^{-7} M). After cumene hydroperoxide (3×10^{-4} M) incubation, the precontraction by methacholine (3×10^{-7} M) has been diminished (according to the results described above). The potency of β-adrenoceptor agonists to relax the tracheal smooth muscle is inversely related to the strength of contraction (Kenakin 1985). We would thus expect to observe an increased (−)-isoprenaline-induced relaxation relative to the precontraction when the β-adrenoceptor response would be unaffected after cumene hydroperoxide (3×10^{-4} M), according to the phenomena described by Kenakin (1985). However, as indicated we did not see a change in (−)-isoprenaline induced response. This suggests that the β-adrenoceptor response is diminished by incubation with cumene hydroperoxide (3×10^{-4}M). Both parasympathetic and sympathetic receptor responses have been changed after incubation with cumene hydroperoxide (3×10^{-4} M), and thus the autonomic balance seems to be affected.

The β-adrenoceptor response of denuded tracheal strips increased after cumene hydroperoxide (3×10^{-5} M) exposure, while no change in precontraction was observed. Van Amsterdam et al (1989) reported that the ability of the β-adrenoceptor agonists to relax tracheal tissue is not only dependent on the strength of the contraction, but depends on the amount of inositol phosphates formed after muscarinic or histamine receptor stimulation. The observed increase in β-adrenoceptor response after pretreatment with cumene hydroperoxide (3×10^{-5} M), might, therefore, be due to a decreased formation of inositol phosphates by muscarinic receptor stimulation.

The protective role of the epithelial layer against oxidative stress has been emphasized by this study because the β-adrenoceptor response of denuded tracheal strips (after cumene hydroperoxide pretreatment with 3×10^{-5} M) was affected while the β-adrenoceptor response of normal (intact epithelium) tracheal strips was still intact.

The destruction and removal of epithelium and change in autonomic balance are two characteristics of asthmatic lungs (Houston et al 1953; Chung 1986; Lemanske & Kaliner 1990). Reactive oxygen species (for instance formed by cumene hydroperoxide) seem to induce similar pathological conditions. We therefore suggest that protection against reactive oxygen species may be useful in therapy of asthmatic patients.

In summary, we report here that the β-adrenoceptor response of rat tracheal strips diminishes after mechanical removal of the epithelial layer. Cumene hydroperoxide causes a destruction and displacement of the rat tracheal epithelium. A diminished muscarinic and β-adrenoceptor response was observed after cumene hydroperoxide treatment. Especially, an increase of β-adrenoceptor response, due to a decreased muscarinic receptor response, was expected after cumene hydroperoxide (3×10^{-4} M) treatment, but the β-adrenoceptor response was unaffected. Both parasympathetic and sympathetic receptor responses have been changed after cumene hydroperoxide treatment. The protective role of the epithelial layer against oxidative stress has been underlined.

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