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Role of the epithelium in opposing H_2O_2-induced modulation of acetylcholine-induced contractions in rabbit intrapulmonary bronchiole

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1 The role played by the epithelium in H_2O_2-induced modulation of the mechanical responses induced by acetylcholine (ACh) in rabbit intrapulmonary bronchioles was investigated in epithelium-intact and -denuded strips.

2 When ACh (3 μM) was applied intermittently, H_2O_2 (30 μM) enhanced the ACh-induced contractions in epithelium-intact strips. In contrast, in epithelium-denuded strips H_2O_2 (30 μM) inhibited such contractions. At higher concentrations, H_2O_2 concentration-dependently attenuated the ACh-induced contractions in both epithelium-intact and -denuded strips, its action being more potent in the latter strips than in the former.

3 Diclofenac (a cyclo-oxygenase inhibitor; 3 μM) reduced the H_2O_2-induced enhancement of ACh-contractions in epithelium-intact strips but had no effect on the H_2O_2-induced inhibition in epithelium-denuded strips. N^G-nitro-L-arginine did not alter the effect of H_2O_2 on ACh-induced contractions in epithelium-intact strips.

4 Catalase (500 u ml^{-1}) completely blocked both H_2O_2-induced effects on ACh-contractions (enhancement and inhibition). Neither superoxide dismutase (200 u ml^{-1}) nor deferoxamine (0.5 mM) had any effect on H_2O_2-induced inhibition in epithelium-denuded strips.

5 Aminotriazole (an inhibitor of catalase; 50 mM) significantly potentiated the H_2O_2-induced inhibition of ACh-contractions in epithelium-intact strips but not in epithelium-denuded strips.

6 The density ratio for catalase (epithelium-intact over -denuded strips) analysed by Western blot was about 2.1, suggesting that epithelium contains more catalase than smooth muscle.

7 It is concluded that in rabbit intrapulmonary bronchioles, H_2O_2 has dual actions on ACh-contractions. It is suggested that the epithelium may act as a powerful biochemical barrier *via* both the action of catalase (scavenging H_2O_2) and the release of bronchoconstrictor prostaglandins, thus attenuating the H_2O_2-induced modulation of ACh-contractions.
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Keywords: Hydrogen peroxide; airway smooth muscle; airway epithelium; catalase; aminotriazole; prostaglandin; nitric oxide; asthma

Abbreviations: ACh, acetylcholine; L-NNA, N^G-nitro-L-arginine; PGs, prostaglandins; SOD, superoxide dismutase

Introduction

A variety of mediators released from such inflammatory cells as eosinophil leucocytes, neutrophil granulocytes and alveolar macrophages are thought to play an important role in airway inflammation (Dent *et al*., 1994). Upon activation, these cells release such reactive oxygen species as free radicals (e.g. superoxide anion, hydroxy radical) and oxygen metabolites (e.g. H_2O_2, hypochlorous acid), all of which may be involved in the pathophysiology of asthma (Babior, 1984; Chung, 1986; Cross *et al*., 1987; Barnes, 1989). Of these, H_2O_2 is a ubiquitous reactive oxygen metabolite that readily penetrates cell membranes and is potentially harmful (Schubert & Wilmer, 1991).

The effect of H_2O_2 on airway smooth muscle has been studied in several species. For example, H_2O_2 itself produces a contraction of the airways in guinea-pig (Rhoden & Barnes, 1989; Gupta & Prasad, 1992), rat (Szarek & Schmidt, 1990) and human (Rabe *et al*., 1995). Moreover, it inhibits the ACh-induced contraction of the airways in guinea-pig (Gao & Vanhoutte, 1993) and rabbit (Gupta & Prasad, 1992). It has been suggested that in the main trachea, the epithelium may be the source of dilator and constrictor mediators that act to modulate the H_2O_2-induced contraction; indeed, in guinea-pig trachea the epithelium generates prostaglandins (PGs) in response to H_2O_2 and these may contribute to the H_2O_2-induced modulation of relaxation (Gao & Vanhoutte, 1993) or constriction (Rhoden & Barnes, 1989). Furthermore, in addition to its role as a source of PGs, the epithelium in

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human bronchi may act as a physical or biochemical barrier preventing H_2O_2 from inducing contraction (Rabe *et al*., 1995), although the precise mechanism remains unidentified. The presence of catalase and glutathione peroxidase (scavengers of H_2O_2) has been histochemically established in rat airway epithelial cells (Coursin *et al*., 1992). The epithelial antioxidant defence is thought to be of crucial importance in preventing the initiation or propagation of airway pathology in, for example, adult respiratory distress syndrome, chronic pulmonary emphysema and asthma (Adler *et al*., 1990; Barnes, 1989; Bast *et al*., 1991). However, the mechanism underlying this epithelium-mediated defence against oxygen radicals has not yet been fully clarified in intrapulmonary bronchioles.

In the present study, we set out to clarify the role played by the epithelium during H_2O_2-induced mechanical responses in intrapulmonary bronchioles. To this end, we pharmacologically characterized the alterations in the effects produced by H_2O_2 on ACh-induced contractions when various types of enzyme inhibitors were applied to epithelium-intact and -denuded strips. The part played by cyclo-oxygenase products was assessed by applying diclofenac, a potent inhibitor of cyclo-oxygenase, and that played by catalase by applying aminotriazole, a selective catalase inhibitor (Burke-Wolin & Wolin, 1989; Yusa *et al*., 1987). Furthermore, amounts of catalase present in epithelium-intact and -denuded strips were estimated by Western blot analysis.

Methods

Male Japan White albino rabbits (supplied by Kitayama Labes Co. Ltd, Japan), weighing 1.9–2.5 kg, were anaesthetized by injection of pentobarbitone sodium (40 mg kg^{-1}, i.v.) and then exsanguinated. The protocols used conformed with guidelines on the conduct of animal experiments issued by Nagoya City University Medical School and by the Japanese government (Law no. 105; Notification no. 6), and were approved by The Committee on the Ethics of Animal Experiments in Nagoya City University Medical School. The left lung was rapidly removed and placed in a chamber filled with Krebs solution. The left upper bronchial tree (fourth generation, outer diameter approximately 0.20–0.35 mm) was dissected under a binocular microscope, connective tissues being carefully removed. After a given bronchiole had been cut along its long axis using a small scissors, circularly cut strips (0.15–0.3 mm wide, 0.025–0.03 mm thick, 0.6–0.8 mm long) were prepared, care being taken not to damage the epithelium. In some strips, the epithelium was carefully removed by gentle rubbing of the internal surface of the strip using a small piece of razor blade, as described previously (Itoh *et al*., 1992). Satisfactory ablation of the epithelium was verified histologically (by staining with haematoxylin and eosin).

Recording of mechanical responses

To enable recording of isometric force, a fine circularly cut strip was prepared and transferred to a chamber of 0.6 ml volume mounted horizontally on a microscope. Mechanical responses were measured by attaching the strip to a strain gauge (U-gauge, UL-2 type; Shinko, Tokyo, Japan). The ends of the preparation were fixed using pieces of Scotch double-stick tape (3M Co., St Paul, MN, U.S.A.), one to an anchorage point in the recording chamber and the other to the transducer, as described previously (Itoh *et al*., 1992). A given solution was injected rapidly using a syringe from one end of the chamber and simultaneously aspirated by a pump from the other end, enabling the solution in the chamber to be changed within a few seconds (Figure 1). The resting force (about 2–5 mg) was adjusted to obtain a maximum contraction in response to 3 μM ACh.

After equilibration of the preparation for 90–120 min, 3 μM ACh was repeatedly applied for 3 min at 7-min intervals to epithelium-intact or -denuded strips. Once a constant ACh response had been obtained, the experiment was started. To obtain a concentration-dependent effect of ACh, various concentrations of ACh (0.1–30 μM) were applied for 3 min each at 7-min intervals (in a stepwise manner from low to high concentration). After a control concentration-response relationship for ACh had been obtained, diclofenac (3 μM) was applied for 60 min. Then, the same protocol for ACh-application was repeated in the presence of diclofenac in the same strip.

To observe the effect of H_2O_2, a reproducible contraction to 3 μM ACh was first obtained using the protocol described above (3-min applications at 7-min intervals) in epithelium-intact and -denuded strips. After the control ACh-responses had been recorded, H_2O_2 was applied for 5 min as pretreatment and ACh was subsequently applied in the presence of H_2O_2 (using the protocol described above). The action of H_2O_2 (at concentrations over 30 μM) was irreversible. So, to enable the concentration-dependent effect of H_2O_2 to be examined the above procedure was adopted for each concentration of H_2O_2 using separate preparations obtained from one and the same animal.

When the effect of catalase (500 u ml^{-1}) or superoxide dismutase (SOD, 200 u ml^{-1}) was to be examined in epithelium-intact or denuded strips, each agent was pretreated for 7 min after a constant ACh (3 μM)-response had been obtained. ACh was then applied as before but in the presence of the same agent. Next, H_2O_2 was pretreated for 5 min in the presence of catalase or SOD. Finally, ACh (3 μM) was applied for 3 min at 7-min intervals in the presence of H_2O_2 plus the appropriate agent.

When the effect of deferoxamine (0.5 mM) was to be examined, this agent was pretreated for 60 min after a constant ACh (3 μM)-response had been obtained. ACh was then applied as before but in the presence of deferoxamine. Next, H_2O_2 was pretreated for 5 min in the presence of deferoxamine. Finally, ACh (3 μM) was applied for 3 min at 7-min intervals in the presence of H_2O_2 plus deferoxamine.

When the effect of aminotriazole (50 mM) on H_2O_2-induced responses was to be examined, a reproducible ACh (3 μM)-contraction was first obtained in epithelium-intact and -denuded strips treated with diclofenac (3 μM). Then, aminotriazole was applied for 60 min, followed by a 30-min washout with Krebs solution containing diclofenac (3 μM). ACh (3 μM) was subsequently applied for 3 min at 7-min intervals until a reproducible response was obtained. Finally, H_2O_2 was pretreated for 5 min and ACh was reapplied for 3 min at 7-min intervals in the presence of H_2O_2 plus 3 μM diclofenac.

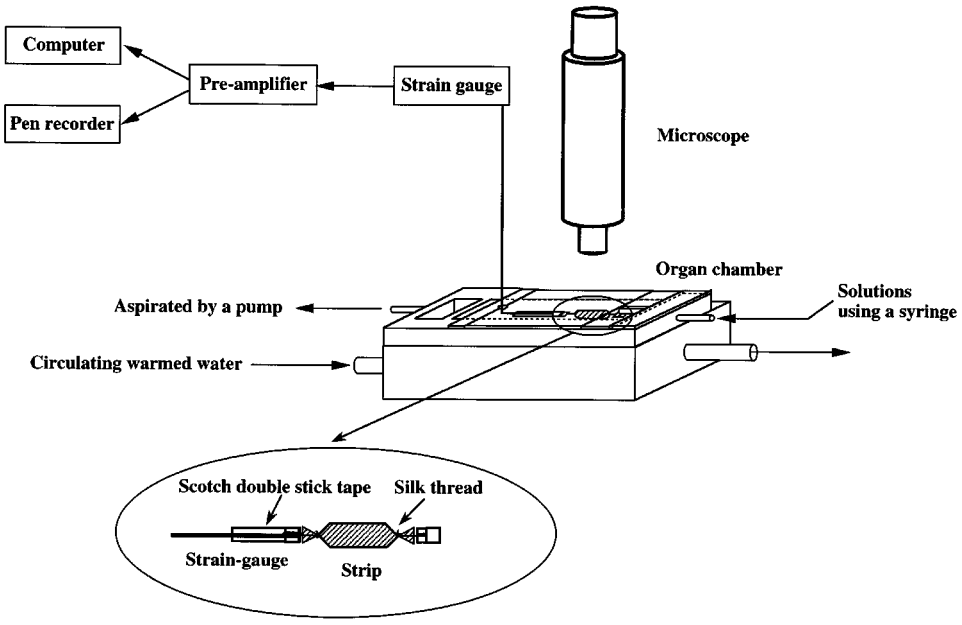

Figure 1 Schematic diagram of experimental system for recording of mechanical response.

Western blot analysis for catalase

Epithelium-intact or -denuded tracheal strips were homogenized using a glass-glass homogenizer at 4°C and sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol and 2% sodium dodecyl sulphate (SDS). After centrifugation, the proteins in the supernatant were quantified using a modified Lowry assay procedure (*DC* Protein Assay Kit; Bio-Rad, CA, U.S.A.), with bovine serum albumin (BSA) being used as standard. Protein samples from epithelium-intact strips or epithelium-denuded strips were heated for 5 min at 100°C in sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% bromophenol blue), electrophoresed along with a positive control (0.1 μg bovine liver catalase; Wako Pure Chemical, Tokyo, Japan) on SDS-7.5% polyacrylamide gel (Ready Gel J; Bio-Rad, CA, U.S.A.) and then transferred to nitrocellulose membranes. The membranes were rinsed with phosphate-buffered saline (PBS; 0.01 M Na_2HPO_4 and 0.25 M NaCl, pH 7.6), then blocked with 4% Block Ace (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) for 2 h at 37°C before being incubated with a primary antibody overnight at 4°C with gentle rocking. Following a washout with PBS, the membranes were incubated for 1 h at room temperature with a secondary antibody in PBS containing 1% BSA. A polyclonal anti-catalase antibody (Biogenesis Ltd, Poole, U.K.) was used as the primary antibody (1 : 500 dilution) and donkey anti-sheep/goat immunoglobulins peroxidase (Binding Site Ltd, Birmingham, U.K.) was used as the secondary antibody (1 : 500 dilution). After final washes with PBS, the signals from the immunoreactive bands were detected by enhanced chemiluminescence using a chemiluminescent detection system (SuperSignal®; West Pico, Pierce, IL, U.S.A.) and Hyperfilm (Amersham Pharmacia Biotech Ltd, Buckinghamshire, U.K.). The density of the protein was measured by densitometric scanning, as described previously (Suzuki & Itoh, 1993).

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. All the solutions used in the present experiments contained guanethidine (5 μM, to prevent sympathetic transmitter release). The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH was maintained at 7.4.

Drugs

The drugs used were diclofenac sodium, deferoxamine mesylate, aminotriazole (Sigma, St. Louis, MO, U.S.A.), acetylcholine hydrochloride (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan), N^G-nitro-L-arginine (L-NNA; Peptide Institute, Minoh, Japan), bovine liver catalase and bovine erythrocyte superoxide dismutase (SOD; Wako Pure Chemical, Tokyo, Japan) and guanethidine (Tokyo Kasei, Tokyo, Japan). The drugs were dissolved in ultra-pure Milli-Q water (Japan Millipore Corp., Tokyo, Japan) to make stock solutions.

Statistics

Values are expressed as mean ± s.e.mean. Multiple comparisons and time-dependent effects were examined by a two-way repeated-measures ANOVA followed by a Bonferroni test for *post hoc* analysis. When means for the same group or two different groups were to be compared, a Student's *t*-test was used (paired and unpaired, respectively). Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effects of H_2O_2 on ACh-induced contractions

In strips from intrapulmonary bronchioles, ACh (0.3–30 μM) produced a phasic, followed by a tonic contraction in a concentration-dependent manner. The maximum force and the pD_2 values were not significantly different whether epithelium was present or not (Table 1; $P>0.1$ in each case, unpaired *t*-test).

In epithelium-intact strips, H_2O_2 (30 μM) enhanced both the phasic and the tonic contraction induced by 3 μM ACh (Figure 2A, $P<0.01$ in each case). When a higher concentration of H_2O_2 (50 μM) was used, it attenuated the tonic contraction in a time-dependent manner ($P<0.05$) but not the phasic contraction ($P=0.10$). H_2O_2 (100 μM) inhibited both the phasic and the tonic contraction ($P<0.001$ in each case). Thus, the magnitude of the inhibition of the ACh-contractions effectively increased as a function of the concentration of H_2O_2. In contrast, in epithelium-denuded strips H_2O_2 (at 30 μM but not at 10 μM) attenuated both of the ACh-induced responses as a function of time ($P<0.001$ in each case). With an increase in the concentration to 50 μM, this agent had a more powerful attenuating effect (vs the effect of 30 μM H_2O_2, $P<0.003$ in each case, Figure 2B). In both epithelium-intact and -denuded strips, the ACh-contraction had not fully recovered 20 min after washout of H_2O_2 when a high concentration of H_2O_2 (>30 μM) had been used (not shown). The inhibitory action of 30 μM H_2O_2 was greater on the ACh-induced tonic contraction than on the ACh-induced phasic contraction in epithelium-denuded strips ($P<0.05$). Furthermore, H_2O_2 was more potent at inducing inhibition in epithelium-denuded strips than in epithelium-intact strips (Figure 2). For example, at 55 min after the application of 50 μM H_2O_2, the ACh-induced tonic contraction was inhibited by $46.2\pm7.9\%$ ($n=6$) and $99.7\pm0.3\%$ ($n=6$) of the initial contraction in epithelium-intact and -denuded strips, respectively ($P<0.001$).

Effect of diclofenac on the action of H_2O_2

Diclofenac (3 μM) had no significant effect on the ACh-induced phasic or tonic contraction in the absence of H_2O_2 in epithelium-intact or -denuded strips ($P>0.1$ in each case, paired *t*-test, Table 1). In epithelium-intact strips in the presence of diclofenac, H_2O_2 (30 μM) had a more transient enhancing effect on the phasic and tonic contractions induced by 3 μM ACh than it did in the absence of diclofenac ($P<0.05$ in each case, Figure 3A). In epithelium-denuded strips (Figure 3B), diclofenac did not significantly modulate the H_2O_2 (30 μM)-induced inhibition of either the phasic or the tonic contraction induced by 3 μM ACh ($P>0.7$ in each case).

The modulating action of L-NNA (0.1 mM) on the effect of H_2O_2 (50 μM) on the ACh-induced contractions was examined in epithelium-intact strips treated with 3 μM diclofenac. L-NNA had no effect on either the phasic or the tonic contraction induced by 3 μM ACh (for the phasic response, 0.99 ± 0.01 times control, $P=0.10$; for the tonic response, 1.03 ± 0.02 times control, $P=0.34$, $n=5$). L-NNA did not significantly modify the inhibitory effect of 50 μM H_2O_2 on the ACh-contractions ($P>0.4$ in each case).

Effects of catalase, SOD and deferoxamine on the action of H_2O_2

In epithelium-intact and -denuded strips, catalase (500 u ml^{-1}) had no significant effect on either the phasic or the tonic contraction induced by 3 μM ACh ($P>0.2$ in each case, $n=4$). However, catalase abolished the H_2O_2 (30 μM)-induced enhancement of the ACh-contractions in epithelium-intact strips ($P<0.002$ in each case, Figure 4A). Likewise, in epithelium-denuded strips, catalase (500 u ml^{-1}) blocked the H_2O_2 (50 μM)-induced effect (inhibition) on both the phasic and the tonic contraction induced by 3 μM ACh ($P<0.05$ in each case, Figure 4B).

SOD (200 u ml^{-1}) had no significant effect on either the phasic or the tonic contraction induced by 3 μM ACh in epithelium-denuded, diclofenac-treated strips (for phasic response, 0.98 ± 0.02 times control, $P=0.25$; for tonic response, 0.98 ± 0.03 times control, $P=0.36$, $n=4$). Moreover, SOD did not significantly modify the inhibitory effect of 20 μM H_2O_2 on the ACh-contractions in epithelium-denuded, diclofenac-treated strips ($P>0.2$ in each case, Figure 5A).

In contrast, deferoxamine (0.5 mM) significantly attenuated both the phasic and tonic contractions induced by 3 μM ACh in the absence of H_2O_2 in epithelium-denuded, diclofenac-treated strips (for phasic response, 0.79 ± 0.02 times control, $P<0.001$; for tonic response, 0.78 ± 0.02 times control, $P<0.001$, $n=5$). Deferoxamine did not significantly modify the inhibition by 20 μM of H_2O_2 of the ACh-contractions ($P>0.5$ in each case, Figure 5B).

Effect of aminotriazole on the actions of H_2O_2

In epithelium-intact, diclofenac-treated strips, neither the phasic nor the tonic contraction induced by 3 μM ACh was significantly modified by 50 mM aminotriazole (for phasic response, 1.13 ± 0.14 times control response observed before application of aminotriazole, $P=0.37$; for tonic response,

Table 1 Effect of diclofenac on the phasic and tonic contractions induced by 3 μM ACh

	Control				*Diclofenac-treated*			
	pD_2		*Maximum response* (mg)		*pD_2*		*Maximum response* (mg)	
	Phasic	*Tonic*	*Phasic*	*Tonic*	*Phasic*	*Tonic*	*Phasic*	*Tonic*
Epithelium-intact	5.93 ± 0.11	5.82 ± 0.09	33.4 ± 5.7	28.4 ± 5.2	5.95 ± 0.13	5.95 ± 0.10	33.0 ± 6.2	25.3 ± 5.2
Epithelium-denuded	5.59 ± 0.02	5.48 ± 0.03	36.2 ± 7.9	28.3 ± 5.5	5.53 ± 0.02	5.55 ± 0.11	35.3 ± 7.1	28.3 ± 5.5

ACh (0.1–30 μM) was applied for 3 min at 7-min intervals (from low to high concentration). After recording a control series of ACh responses, diclofenac (3 μM) was applied for 60 min and ACh (0.1–30 μM) was then reapplied in the presence of diclofenac. Thus, the pD_2 value and the ACh-induced maximum response were obtained from one and the same strip. The maximum amplitude of contraction was obtained by application of 30 μM ACh. For each group (epithelium-intact or -denuded), the number of strips used was six (from three animals). Data are expressed as mean$\pm$s.e.mean.

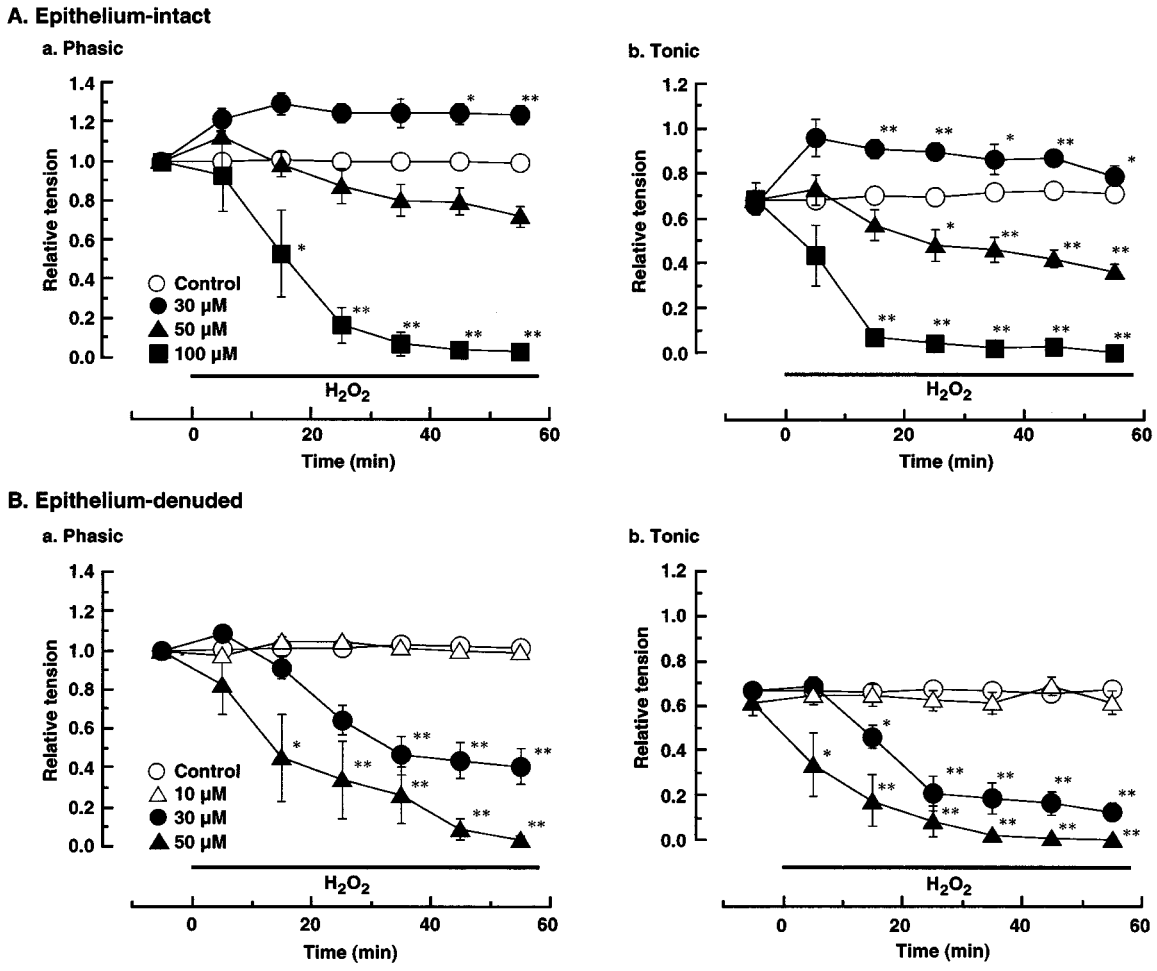

Figure 2 Concentration-dependent effects of H_2O_2 on the phasic and tonic contractions induced by 3 μM ACh in epithelium-intact and -denuded strips from rabbit intrapulmonary bronchioles. (A) Epithelium-intact strips: Aa, ACh-induced phasic contraction; Ab, ACh-induced tonic contraction. (B) Epithelium-denuded strips: Ba, ACh-induced phasic contraction; Bb, ACh-induced tonic contraction. ACh (3 μM) was applied for 3 min at 7-min intervals. H_2O_2 was applied as indicated by the bar. Concentration of H_2O_2 is indicated in the keys (note different concentrations between A and B). The ACh-induced tonic contraction was measured at 3 min after the start of the ACh application. The maximum amplitude of phasic contraction induced by 3 μM ACh before application of H_2O_2 was normalized as a relative tension of 1.0 for each strip. Mean of data from 4–8 strips, with s.e.mean shown by vertical line. The standard error bars for a number of the points in the graphs fall within the symbol being used. $^*P<0.05$, $^{**}P<0.01$ vs Control.

1.25 ± 0.18 times control, $P=0.19$, $n=6$). Similarly, in epithelium-denuded, diclofenac-treated strips, neither the phasic nor the tonic contraction induced by 3 μM ACh was significantly modified by aminotriazole (for phasic response, 1.02 ± 0.08 times control, $P=0.83$; for tonic response, 1.05 ± 0.08 times control, $P=0.58$, $n=5$).

In epithelium-intact, diclofenac-treated strips, the inhibitory effect of H_2O_2 (50 μM) on the ACh-contractions was greater in strips treated with aminotriazole than in those not treated with this agent ($P<0.001$ in each case, Figure 6A). In contrast, in epithelium-denuded, diclofenac-treated strips, the inhibitory effects of H_2O_2 (20 μM) on the ACh-contractions were not significantly altered by aminotriazole ($P>0.6$ in each case, Figure 6B).

Western blot analysis of catalase content of epithelium-intact and -denuded strips

In the Western blot analysis shown in Figure 7, lane 1 shows the positive control (catalase from bovine liver; amount of protein applied 0.1 μg), while lanes 2 and 3 show the immunoreactive bands from epithelium-denuded and -intact strips, respectively (amounts of protein applied 75 and 50 μg, respectively). The immunoreactive band in lane 2 was less dense than that in lane 3. The density ratio (immunoreactive band from epithelium-intact strips over that from epithelium-denuded strips) was 2.14 ± 0.58 ($n=4$) when the applied protein concentrations were normalized.

Discussion

In the present experiments, we found that H_2O_2 had dual actions on ACh-induced contractions in epithelium-intact strips from rabbit intrapulmonary bronchioles (*viz.* enhancement at a low concentration (30 μM) and inhibition at higher concentrations). In contrast, in epithelium-denuded strips H_2O_2 (>10 μM) concentration-dependently attenuated the ACh-induced contraction. The inhibitory effect of H_2O_2 was greater in epithelium-denuded strips than in epithelium-intact

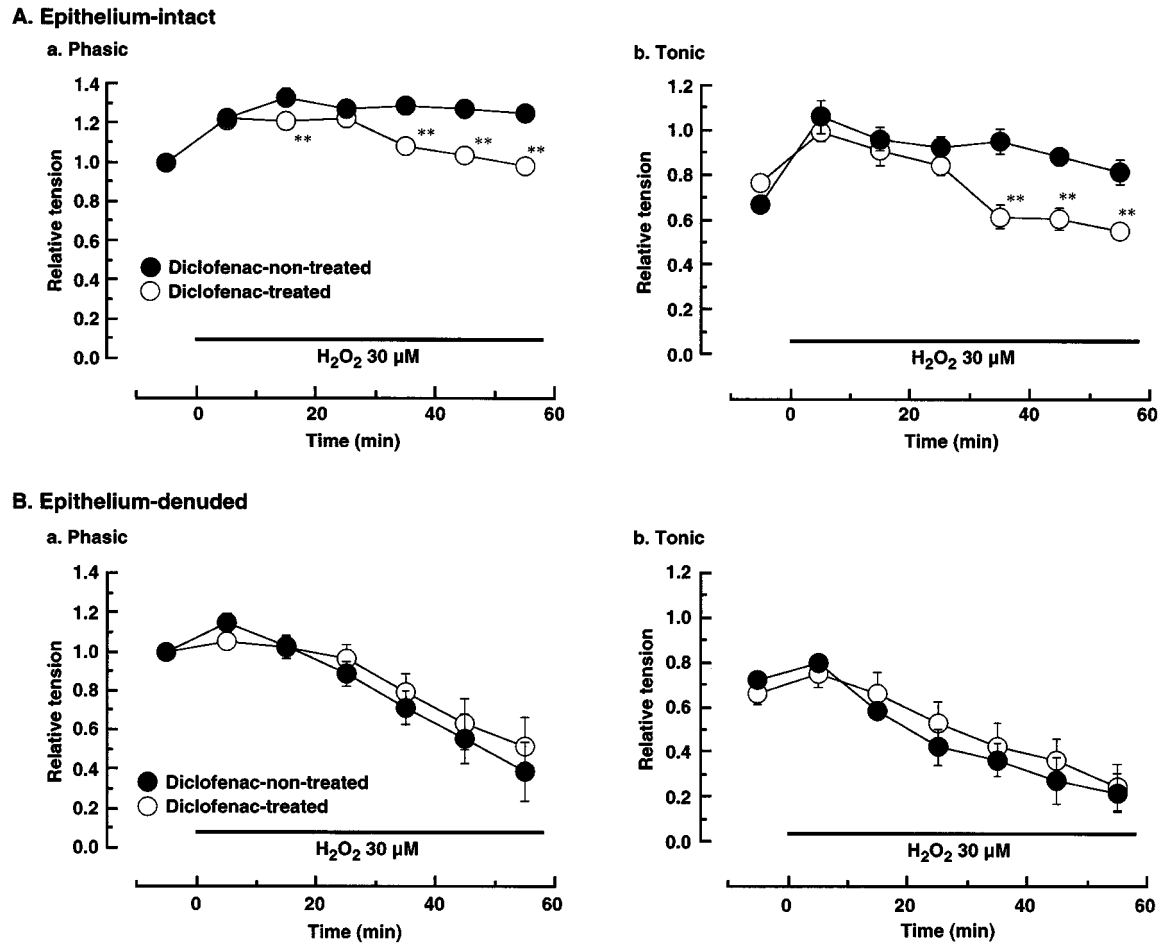

Figure 3 Effect of H_2O_2 on the phasic and tonic contractions induced by 3 μM ACh in epithelium-intact and -denuded strips in the presence and absence of diclofenac. (A) Epithelium-intact strips. Aa, Ach-induced phasic contraction; Ab, ACh-induced tonic contraction. (B) Epithelium-denuded strips: Ba, ACh-induced phasic contraction; Bb, ACh-induced tonic contraction. ACh (3 μM) and H_2O_2 (30 μM) were applied as in Figure 2. The maximum amplitude of phasic contraction induced by 3 μM ACh before application of H_2O_2 was normalized as a relative tension of 1.0 for each strip. When used, diclofenac (3 μM) was pretreated for 60 min before application of H_2O_2 and was present throughout the experiment. Mean of data from 4–6 strips, with s.e.mean shown by vertical line. The standard error bars for a number of the points in the graphs fall within the symbol being used. $^{**}P<0.01$ vs Diclofenac-non-treated.

strips when the effects were compared at a given concentration. Both the enhancing action of H_2O_2 (in epithelium-intact strips) and its inhibitory action (in epithelium-denuded strips) were completely blocked by catalase. Neither superoxide dismutase (SOD, to inactivate superoxide) nor deferoxamine (to inhibit the generation of the hydroxy radical) modified the H_2O_2-induced inhibition of ACh-contractions in epithelium-denuded, diclofenac-treated strips. These results indicate that the effects of H_2O_2 seen in the present experiments on the rabbit bronchiole were indeed due to actions of H_2O_2 itself.

It has been suggested that the epithelium modulates the responses of the underlying smooth muscle through the release of such inhibitory factors as epithelium-derived relaxing factor (NO), prostaglandins (PGs) and hyperpolarizing factor (Flavahan *et al*., 1985; Goldie *et al*., 1990; Morrison *et al*., 1990; Gao & Vanhoutte, 1993). It has been found that in the trachea, removal of the epithelium either augments the H_2O_2-induced contraction (guinea-pig: Rhoden & Barnes, 1989; Gao & Vanhoutte, 1993; rat: Szarek & Schmidt, 1990) or attenuates the H_2O_2-induced relaxation of the ACh-contraction (guinea-pig: Gao & Vanhoutte, 1993; dog: Gao & Vanhoutte, 1992; rabbit: Gupta & Prasad, 1992). Some possible mechanisms underlying these modulatory actions of the epithelium are: (i) the epithelium may act as a physical barrier to H_2O_2 (although in fact the epithelium shields only one side of the present preparation), (ii) the epithelium may release relaxing substances in response to H_2O_2 and thus act as a biochemical barrier serving to modulate the response of the smooth muscle or (iii) the epithelium may serve as a biochemical barrier through a direct inactivation of H_2O_2.

N^G-monomethyl-L-arginine, an inhibitor of NO synthase, attenuates the H_2O_2-induced relaxation of the ACh-contraction in the trachea (guinea-pig: Gao & Vanhoutte, 1993; rabbit: Gupta & Prasad, 1992). In contrast, in the present experiments we found that L-NNA, another type of inhibitor of NO synthase, had no effect on either the ACh-induced

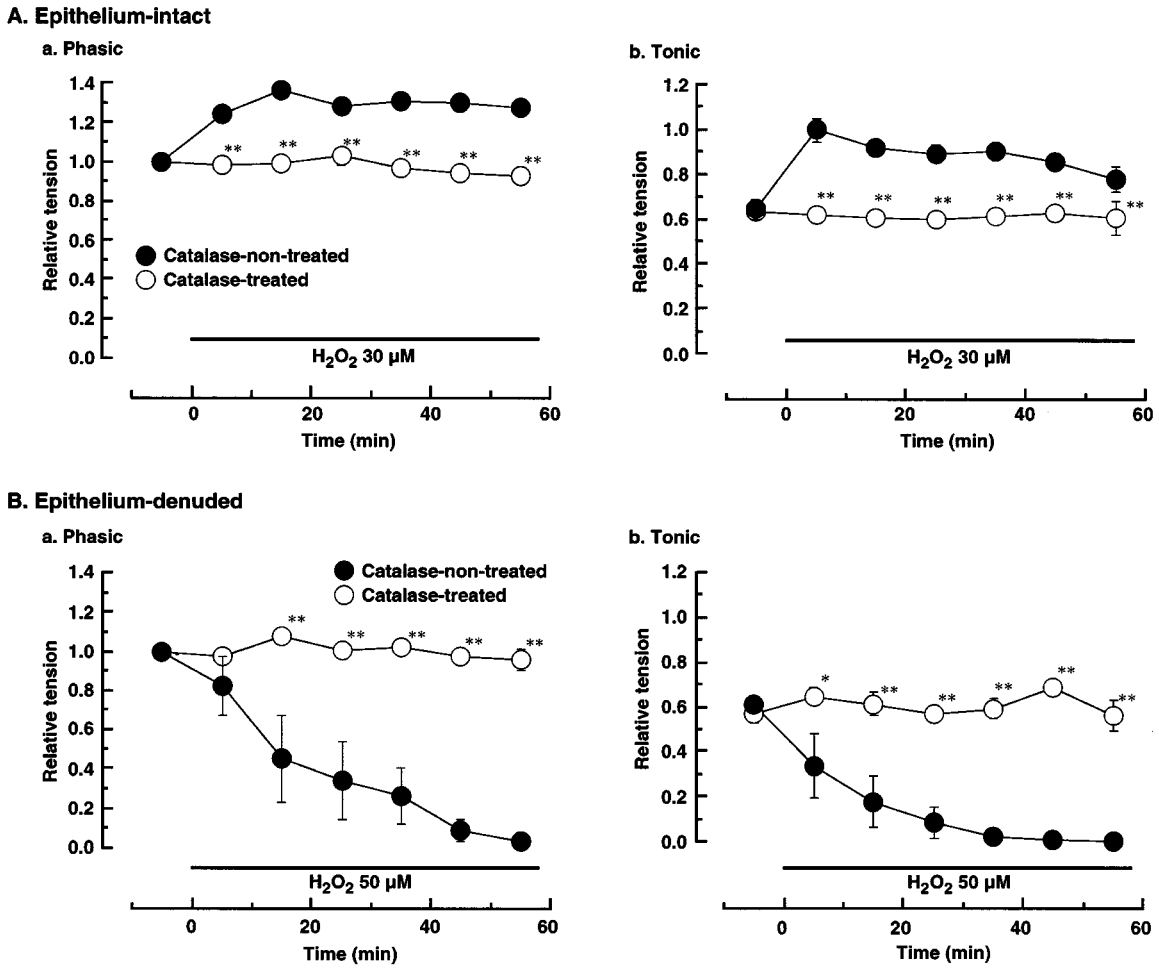

Figure 4 Effect of catalase on the action of H_2O_2 on the contractions induced by 3 μM ACh in epithelium-intact and -denuded strips. (A) Epithelium-intact strips: Aa, ACh-induced phasic contraction; Ab, ACh-induced tonic contraction. (B) Epithelium-denuded strips: Ba, ACh-induced phasic contraction; Bb, ACh-induced tonic contraction. ACh (3 μM) and H_2O_2 (30 or 50 μM) were applied as in Figure 2. The maximum amplitude of phasic contraction induced by 3 μM ACh before application of H_2O_2 was normalized as a relative tension of 1.0 for each strip. When used, catalase (500 u ml^{-1}) was pretreated for 7 min and was present throughout the experiment. Mean of data from four strips, with s.e.mean shown by vertical line. The standard error bars for a number of the points in the graphs fall within the symbol being used. $^*P<0.05$, $^{**}P<0.01$ vs Catalase-non-treated.

contractions (in the absence of H_2O_2) or the H_2O_2-induced inhibition of ACh-contractions in the epithelium-intact rabbit bronchiole. We also found that sodium nitroprusside (10 and 30 μM), an NO donor, did not modify the ACh-contractions in epithelium-denuded strips from the rabbit bronchiole ($n=3$; T. Asano, unpublished observations). These results may suggest that the lack of an effect attributable to epithelium-derived NO may be due to NO having a weak action in the rabbit intrapulmonary bronchiole, even if it is released from the bronchiolar epithelium in response to ACh or H_2O_2. Furthermore, in unpublished preliminary experiments we found that H_2O_2 produced only a small membrane hyperpolarization that was not significantly different between epithelium-intact strips (-3.8 ± 0.4 mV, $n=4$) and epithelium-denuded strips (-3.5 ± 0.5 mV, $n=4$, $P>0.1$). These results suggest that in the rabbit intrapulmonary bronchiole, neither NO nor hyperpolarizing factor derived from the epithelium play a significant part in modulating H_2O_2-induced mechanical responses.

It is well known that oxidants increase phospholipase A_2 activity, which in turn increases the release of arachidonic acid and the synthesis of PGs in the pulmonary endothelium (Chakraborti *et al*., 1989). The effects of H_2O_2 on mechanical responses in the trachea are attenuated by indomethacin (an inhibitor of cyclo-oxygenase) in species such as guinea-pig, rat, rabbit and dog (Rhoden & Barnes, 1989; Szarek & Schmidt, 1990; Gao & Vanhoutte, 1992; 1993). Further, a production of PGE_2 and PGI_2 in response to H_2O_2 has been identified in both epithelium-intact and -denuded preparations obtained from guinea-pig and canine airways (Grunstein *et al*., 1991; Gao & Vanhoutte, 1992; 1993), suggesting that PGs synthesized in the epithelium as well as in smooth muscle cells may play a part in the modulation of H_2O_2-induced mechanical responses in the airways. In our epithelium-intact strips, diclofenac (a potent inhibitor of cyclo-oxygenase) attenuated the H_2O_2 (30 μM)-induced enhancement of the ACh-contractions, although this agent had no significant effect on ACh-contractions in the absence of

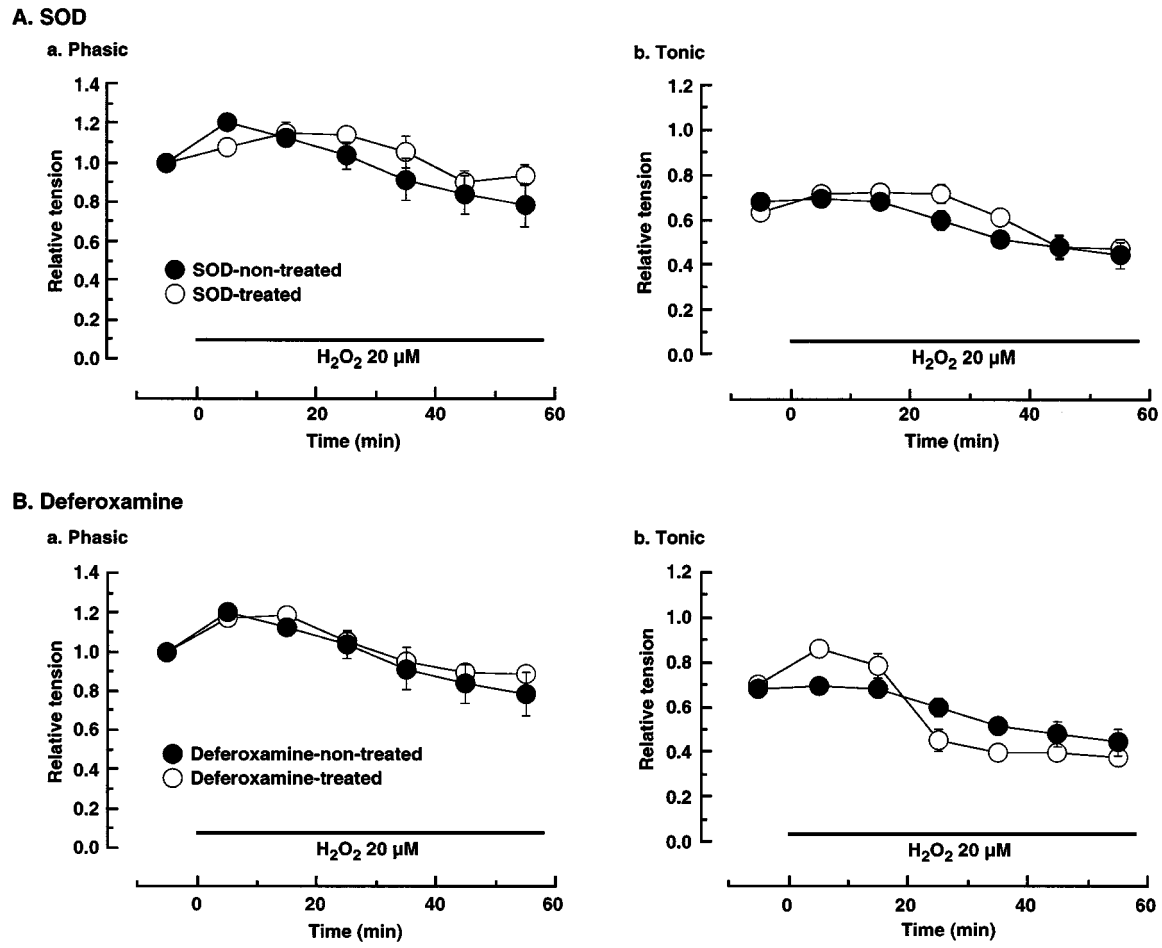

Figure 5 Effect of SOD (A) or deferoxamine (B) on the effect of H_2O_2 on the contractions induced by 3 μM ACh in epithelium-denuded strips. (A) Effect of SOD: Aa, ACh-induced phasic contraction; Ab, ACh-induced tonic contraction. When used, SOD (200 u ml^{-1}) was pretreated for 7 min and was present throughout the experiment. (B) Effect of deferoxamine: Ba, ACh-induced phasic contraction; Bb, ACh-induced tonic contraction. When used, deferoxamine (0.5 mM) was pretreated for 60 min and was present throughout the experiment. ACh (3 μM) and H_2O_2 (20 μM) were applied as in Figure 2. The maximum amplitude of phasic contraction induced by 3 μM ACh before application of H_2O_2 was normalized as a relative tension of 1.0 for each strip. Mean of data from four strips, with s.e.mean shown by vertical line. The standard error bars for a number of the points in the graphs fall within the symbol being used.

H_2O_2. However, in epithelium-denuded strips diclofenac modified neither the ACh-contractions induced in the absence of H_2O_2 nor the H_2O_2 (30 μM)-induced inhibition of the ACh-contractions. These results suggest that in the rabbit intrapulmonary bronchiole, bronchoconstrictor PGs derived from the epithelium may contribute to the H_2O_2-induced enhancement of the ACh-contractions.

It has been suggested that the epithelial cells covering the airway smooth muscle may be an important source of an antioxidant enzyme that acts to protect the respiratory tract (Cohn *et al*., 1994). Both catalase and glutathione peroxidase (scavengers of H_2O_2) are heterogeneously distributed throughout lung tissues (Freeman & Crapo, 1982; Coursin *et al*., 1992), although in cultured guinea-pig epithelial cells, catalase seems to play a more important role in scavenging H_2O_2 than the glutathione redox cycle (Cohn *et al*., 1994). In the present experiments, we found that the inhibitory actions of H_2O_2 on the ACh-contractions were greatly potentiated in the absence of the epithelium. Catalase completely blocked the inhibitory action of a high concentration of H_2O_2 (50 μM) on ACh-contractions in epithelium-denuded strips. Furthermore, aminotriazole (a selective inhibitor of catalase; Burke-Wolin & Wolin, 1989; Yusa *et al*., 1987) significantly potentiated the inhibitory actions of H_2O_2 on ACh-contractions in epithelium-intact, but not in epithelium-denuded strips, although we do not yet know the reason for this difference. Moreover, the amount of catalase present in epithelium-intact strips appears to be much higher than that in epithelium-denuded strips. These results suggest that the bronchiolar epithelium may serve as a biochemical barrier against H_2O_2 *via* a direct inactivation of this agent by catalase.

In conclusion, it is suggested that in the rabbit bronchiole, the epithelium provides a powerful biochemical barrier against H_2O_2 *via* an action of catalase that effectively inactivates this oxygen metabolite. It is also suggested that in response to H_2O_2, the epithelium produces bronchoconstrictor PGs that serve to counteract the inhibitory effect of this agent on ACh-induced contractions. The implication of the above suggestions is that by exerting these actions, the

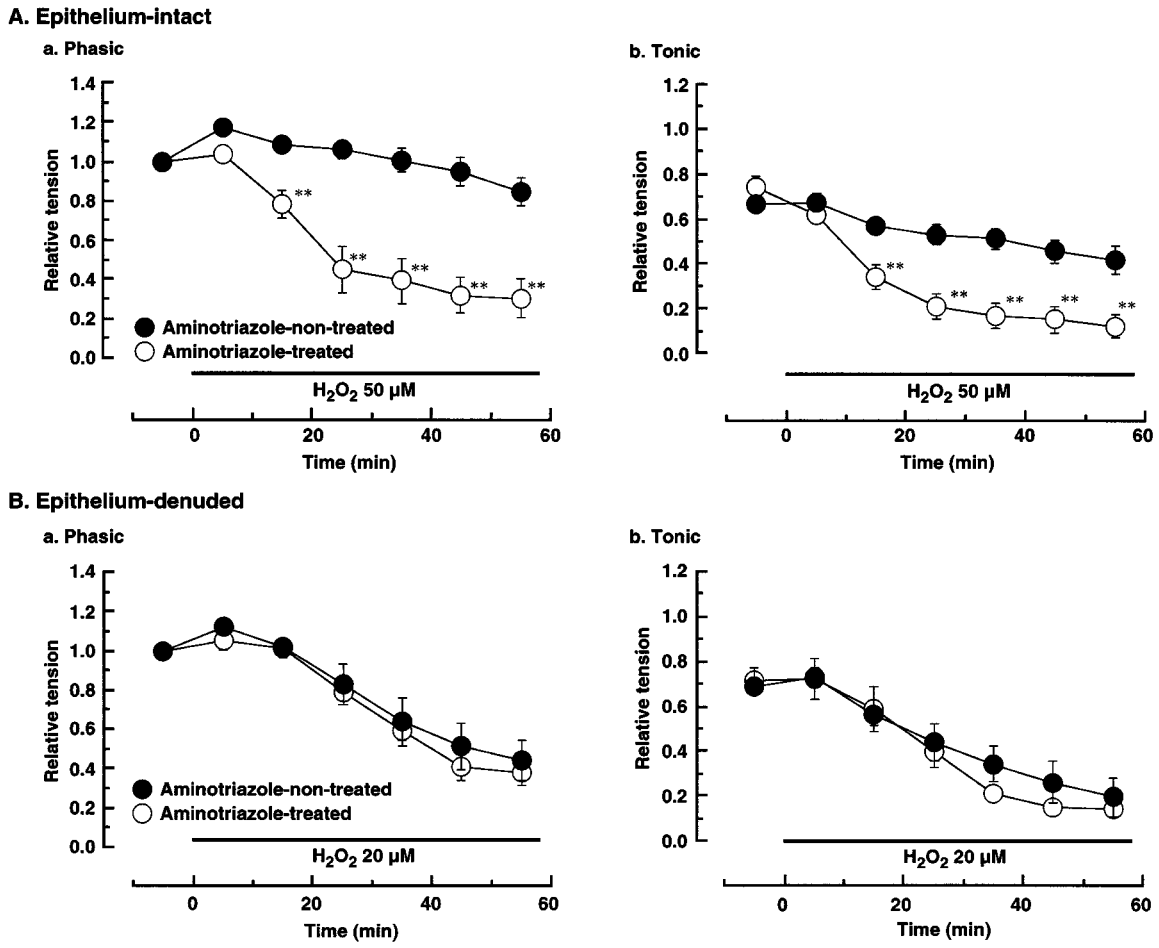

Figure 6 Effect of H_2O_2 on ACh-induced contractions in epithelium-intact (A) and -denuded (B) strips treated or not treated with aminotriazole. (A) Epithelium-intact strips treated with 3 μM diclofenac: Aa, ACh-induced phasic contraction. Ab, ACh-induced tonic contraction. (B) Epithelium-denuded strips treated with 3 μM diclofenac: Ba, ACh-induced phasic contraction; Bb, ACh-induced tonic contraction. When used, aminotriazole (50 mM) was applied for 60 min, followed by a 30-min washout. In all experiments, diclofenac (3 μM) was pretreated for 60 min and was present throughout. ACh (3 μM) and H_2O_2 (20 or 50 μM) were applied as in Figure 2. The maximum amplitude of phasic contraction induced by 3 μM ACh before application of H_2O_2 was normalized as a relative tension of 1.0 for each strip. Mean of data from 5–7 strips, with s.e.mean shown by vertical line. The standard error bars for a number of the points in the graphs fall within the symbol being used. $^{**}P<0.01$ vs Aminotriazole-non-treated.

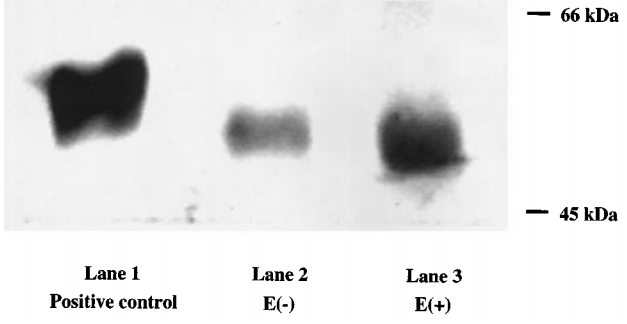

Figure 7 Western blot analysis of catalase content of epithelium-intact and -denuded rabbit tracheae. Homogenates of rabbit tracheal strips with or without epithelium (E(+) and E(−), respectively) were separated by electrophoresis (7.5% SDS–PAGE gel). After transfer to a nitrocellulose membrane, immunoblotting was performed using polyclonal antibodies to catalase (with enhanced chemiluminescence detection). Lane 1, positive control using bovine liver catalase (0.1 μg protein lane^{-1}); lane 2, E(−) (75 μg protein lane^{-1}; lane 3, E(+) (50 μg protein/lane).

epithelium may play a significant role in the rabbit bronchiole's defence against H_2O_2. At present, we think it unwise to speculate about the functional role played by these actions of the bronchiolar epithelium in physiological and pathophysiological situations. However, the epithelium-mediated modulating action described here appears to be a powerful one and, if only for that reason, we should expect studies of its functional role to be well worthwhile.

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